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(71) Applicant: GIST-BROCADES N.V. [NL/NL]; Wateringseweg 1, P.O. Box 1, NL-2600 MA Delft (NL).

(72) Inventors: VAN EEKELEN, Christiaan, Albertus, Gerardus ; Bachplaats 14, NL-2661 HD Bergschenhoek (NL). VAN DER LAAN, Johannes, Cornelis ; J. Jongkindstraat 81/1, NL-1062 CP Amsterdam (NL). MULLENERS, Leonardus, Johannes, Sofie, Marie ; Hoeksestraat 42, NL-5121 SV Rijen (NL).

(74) Agents: HUYGENS, Arthur, Victor et al.; Gist-Brocades N.V., Patents and Trademarks Department, Wateringseweg 1, P.O. Box 1, NL-2600 MA Delft (NL).

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(54) Title: STABLE GENE AMPLIFICATION IN CHROMOSOMAL DNA OF PROKARYOTIC MICROORGANISMS

(57) Abstract

Transformed prokaryotic hosts are provided comprising two or more copies of a DNA sequence stably maintained in their chromosome, said sequence comprising a gene encoding a polypeptide of interest, wherein said copies are separated by endogenous chromosomal DNA sequences. Methods are also provided for producing said transformed host strains. Said transformed host strains are capable of increased production of the polypeptide of interest compared to host strains which already produce said polypeptide. Preferred host strains are *Bacillus* novo species PB92 which produces a high-alkaline proteolytic enzyme and *Bacillus licheniformis* T5 which produce a thermostable alpha-amylase, and mutants and variants of said strains. Preferred polypeptide encoding genes are the protease encoding gene originating from *Bacillus* PB92 and the alpha-amylase encoding gene originating from *Bacillus licheniformis* strain T5.

STABLE GENE AMPLIFICATION IN CHROMOSOMAL DNA
OF PROKARYOTIC MICROORGANISMS

INTRODUCTION

5 Technical Field

The field of this invention relates to prokaryotic
cells in which stable gene amplification is obtained by
scattered non-tandem integration of at least two copies of a
10 defined DNA sequence into the chromosome of said prokaryotic
cell.

Background

15   Bacilli have been widely used for the production of
industrially important enzymes such as alpha-amylase, neutral
protease and alkaline or serine proteases (cf. Debabov, "The
Industrial Use of Bacilli", in: The Molecular Biology of
Bacilli, Acad. Press, New York, 1982). Improvement of
20 production of Bacillus enzymes can be achieved both by
classical genetic techniques, such as mutation and subsequent
selection, and by modern molecular biological techniques. In
the latter case, several ways of obtaining high levels of
expression of homologous and heterologous genes in certain
25 prokaryotic and eukaryotic microorganisms by genetic
engineering have been well documented.
          One of the approaches to achieve high level
expression of a gene is to provide the gene with efficient
regulatory sequences. Another approach, often used in
30 combination with the first approach, is to increase the copy
number of the gene in question. Amplification is primarily
achieved by inserting the gene into a multicopy extrachromo-
somal DNA molecule such as a plasmid. However, a significant
drawback of using plasmids as vectors for expressing and
35 amplifying genetic information has been their instability. For
large scale use, stability of the amplified gene is a pre-
requisite for maintaining high level production of the

expression product encoded by the amplified gene, as many cell
divisions have to take place before sufficient biomass is
formed for obtaining substantial product formation.

Instability is encountered in two forms:
5 segregational instability, where loss of the plasmid occurs
during cultivation; and structural instability, where a part
of the plasmid is deleted. Segregational instability can
occur, for example, when a host cell is harbouring a vector
carrying a gene that is overexpressed. Generally there will be
10 selective pressure towards cells that have lost the capacity
to overexpress the gene, since overexpression is an unfavor-
able property for the transformed host cell. A large amount of
metabolic energy is spent on the overexpressed gene product,
which negatively influences the cells' competitiveness (growth
15 rate) with host cells not likewise overexpressing.

A method used to counter segregational instability
is to select for cells containing multicopy plasmids which
carry genes which confer an advantage on the plasmid
containing cell, for example, conferring resistance to an
20 antibiotic and then to add the relevant antibiotic to the
fermentation broth. However, antibiotics are generally not a
useful selection means in large scale commercial production
processes due to regulations concerning the approval of the
fermentation process or the product itself.

25   Another method used to minimize plasmid loss due to
segregational instability is to insert a gene which is
functionally essential for the host cell into the vector
molecule (Ferrari et al., Biotechnology 3 (1985) 1003-1007).
However, this method does not ensure structural stability of
30 the vector.

Techniques used to solve the problem of structural
plasmid instability have included avoiding expression of the
gene during the phase of exponential growth, for example, by
using regulatory sequences such as temperature-sensitive
35 regulatory sequences, and integration of the exogenous DNA
into the host cell chromosome. Other methods used have
included avoiding the use of autonomously replicating vector
molecules and instead using techniques which favor integration

of the introduced DNA into the host cell chromosome.

Methods of achieving integration of foreign DNA
into the host cell chromosome have included homologous recomb-
ination and illegitimate recombination. There are two ways of
5 inserting DNA sequences into specific locations on a chromo-
some by homologous recombination: Campbell-type homologous
recombination and double reciprocal recombination, which are
shown in Figures 1A and 1B, respectively. A third way of
introducing DNA sequences into the chromosome, this method
10 using a two-step replacement mechanism, is shown in Figure 1C.
In principle, a Campbell-type recombination is used, but the
final result is a chromosomal arrangement that contains no
duplicated sequences, and thus no amplifiable unit, in the
recombined part of the chromosome. It therefore resembles a
15 double reciprocal recombination.

Apart from using homologous recombination for the
integration of foreign DNA into the chromosome it is also
possible to integrate DNA by illegitimate recombination.
Integrated vector molecules can be selected for under con-
20 ditions which inhibit autonomous replication of non-integrated
vector molecules. Use of illegitimate recombination for
integration is depicted in Figure 1D. The absence of tandem
duplications in the obtained chromosomal sequence arrangements
make the pathways shown in Figures 1B, C and D preferred for
25 stable introduction of DNA sequences into the genome.
Chromosomally integrated genes have included both homologous
and heterologous genes where the amplification of the
chromosomally integrated DNA has been in a tandem array.
These chromosomally amplified sequences have been reported to
30 be unstable although stability has been reported in some
cases. It is therefore desirable to develop methods whereby
DNA integrated into the chromosome is stably maintained.

Relevant literature

35

Integration of exogenous DNA by homologous recomb-
ination into the chromosome of Bacillus subtilis has been
described by Duncan et al., Proc. Natl. Acad. Sci. USA 75

(1978) 3664-3668 and for <u>Anacystis</u> <u>nidulans</u> by Williams and
Szalay, Gene <u>24</u> (1983) 37-51 and in International Patent
Application WO 84/00381. Integration by homologous recombinat-
ion of a heterologous gene, which cannot be maintained stably
5 when carried on a plasmid vector, into the chromosome of a
microorganism is described in EP-A-0127328.

Amplification of chromosomally integrated genes,
both homologous and heterologous has been documented. See for
example: Saito <u>et</u> <u>al</u>., Proceedings of the Fourth International
10 Symposium on Genetics of Industrial Microorganisms, Kyoto,
Japan, 1982, pp. 125130; Young, J. Gen. Microbiol. <u>130</u> (1984)
1613-1621; Jannière <u>et</u> <u>al</u>., Gene <u>40</u> (1985) 47-55; Sargent and
Bennett, J. Bacteriol. <u>161</u> (1985) 589-595; Gutterson and
Koshland, Proc. Natl. Acad. Sci. USA <u>80</u> (1983) 4894-4898;
15 Hashiguchi <u>et</u> <u>al</u>., Agric. Biol. Chem. <u>49</u> (1985) 545-550;
Wilson and Morgan, J. Bacteriol. <u>163</u> (1985) 445-453; French
Patent Application No. 84.06701; and EP-A-0134048. Spontaneous
amplification in prokaryotic cells has been reported and can
be selected for. See for example the review by Anderson and
20 Roth, Ann. Rev. Microbiol. <u>31</u> (1977) 473-505.

In all cases referred to above, amplification of
chromosomally integrated DNA was in a tandem array. This type
of chromosomal amplification sequence has been reported to be
unstable, although rather good stability was found in some
25 cases, as discussed by Jannière <u>et</u> <u>al</u>., Gene <u>40</u> (1985) 47-55.

Stabilization of naturally occurring amplified
prokaryotic genes due to the presence of other essential genes
between these amplified sequences has been reported. For
example, of the 9 to 10 copies of the ribosomal RNA gene sets
30 occurring in the <u>B</u>. <u>subtilis</u> chromosome, two tandemly located
sets were separated by a cluster of tRNA genes (Wawrousek and
Hansen, J. Biol. Chem. <u>258</u> (1983) 291-298). In other cases,
naturally occurring tandemly repeated ribosomal RNA operons
were deleted, both in <u>E</u>. <u>coli</u> and in <u>B</u>. <u>subtilis</u>, with little
35 effect on the phenotypic properties of the organism: Ellwood
and Momura, J. Bacteriol. <u>143</u> (1980) 1077-1080 and Loughney <u>et</u>
<u>al</u>., J. Bacteriol. <u>154</u> (1983) 529-532, respectively.

Integration of plasmids into the chromosome of <u>B</u>.

- 5 -

subtilis by illegitimate recombination using the vector pE194
has been described by Hofemeister et al., Mol. Gen. Genet. 189
(1983) 58-68 and Prorozov et al., Gene 34 (1985) 39-46.

5   Several genes for extracellular enzymes of bacilli
have been successfully cloned, such as the alpha-amylase genes
of B. amyloliquefaciens (Palva et al., Gene 15 (1981) 43-51),
B. licheniformis (Ortlepp, Gene 23 (1983) 267), B. stearo-
thermophilus (Mielenz et al., Proc. Acad. Sci. USA 80 (1983)
5975-5979; EP-A-0057976) and B. subtilis (Yang et al., Nucleic
10 Acids Res. 11 (1983) 237); the levansucrase gene of B. subti-
lis (Gay et al., J. Bacteriol. 153 (1983) 1424); the neutral
protease encoding g nes of B. stearothermophilus (Fuji et al.,
J. Bacteriol. 156 (1983) 831), B. amyloliquefaciens (Honjo et
al., J. Biotech. 1 (1984) 165) and of B. subtilis (Yang et
15 al., J. Bacteriol. 160 (1984) 115; the serine or alkaline
protease encoding genes of B. subtilis (Wong et al., Proc.
Natl. Acad. Sci. USA 81 (1984) 1184); B. licheniformis (Jacobs
et al., Nucleic Acids Res. 13 (1985) 8913) and B. amylolique-
faciens (Wells et al., Nucleic Acids Res. 11 (1983) 7911).

20   Protoplast transformation for several species of
gram positive microorganisms has been reported. For B. sub-
tilis a protocol for protoplast transformation was described
by Chang and Cohen (Mol. Gen. Genet. 168 (1979) 111-115),
which has been widely used. Similar successful protocols have
25 been described for the transformation of B. megaterium proto-
plasts (Vorobjeva et al., FEMS Microbiol. Letters 7 (1980)
261-263), B. amyloliquefaciens protoplasts (Smith et al.,
Appl. and Env. Microbiol. 51 (1986) 634), B. thuringiensis
protoplasts (Fisher et al., Arch. Microbiol. 139 (1981) 213-
30 217), B. sphaericus protoplasts (McDonald, J. Gen. Microbiol.
130 (1984) 203), and B. larvae protoplasts (Bakhiet et al.,
Appl. and Env. Microbiol. 49 (1985) 577); in the same publica-
tion unsuccessful results were reported for B. popillae. The
protocol was successful for B. polymyxa, B. licheniformis, B.
35 macerans and B. laterosporus but not for B. coagulans, B.
cereus and B. pumilus, even though good protoplast formation
was observed (Mann et al., Current Microbiol. 13 (1986) 131-
135).

- 6 -

Other methods of introducing DNA into protoplasts
include fusion with DNA containing liposomes (Holubova, Folia
Microbiol. 30 (1985) 97), or protoplast fusion using a readily
transformable organism as an intermediate host cell (EP-A-
5 0134048).

SUMMARY OF THE INVENTION

Prokaryotic host cells, and methods for their
10 preparation, are provided which comprise at least two copies
of a DNA sequence encoding a polypeptide of interest stably
integrated into the host cell chromosome. Stable maintenance
of the exogenous DNA sequence is obtained by integrating two
or more copies of the sequence into the host cell chromosome
15 wherein the copies are separated by endogenous chromosomal DNA
sequences.

BRIEF DESCRIPTION OF THE DRAWINGS

20   Figures 1A-D are schematic representations of four
ways for integration of extrachromosomal DNA sequences into
the chromosome of prokaryotic microorganisms.
          T is the target sequence, i.e. DNA sequences present
on chromosome and plasmid, between which homologous
25 recombination can take place.
          S stands for the DNA sequence to be integrated in
the chromosome.
          M stands for a marker gene sequence used for the
selection of recombinant strain.
30   Figures 2A and 2B are schematic representations of
two ways for obtaining stable gene amplification in a
prokaryotic chromosome.
          Figure 3 shows the results of histidine/MOPS gel
electrophoresis performed on supernatant from cultures of B.
35 subtilis DB104 containing pUB110 and pM58, respectively,
compared with several subtilisins:
          lane 1: Carlsberg subtilisin
          lane 2: Bacillus PB92 protease

- 7 -

            lane 3: Bacillus subtilis subtilisin

            lane 4: Bacillus subtilis DB104 (pM58)

            lane 5: Bacillus subtilis DB104 (pUB110)

      Figure 4 shows the restriction map of plasmid pM58.

5 Furthermore, the sequencing strategy is shown in the upper
part of the figure. The arrowed solid lines represent the
fragments cloned in the phage M13 vectors mp10, mp11 and mp18.
The lower part of the figure shows the sequencing strategy
using ten oligonucleotides located at regular distances on the

10 protease gene.

      Figure 5 shows the nucleotide sequence of the
coding strand correlated with the amino acid sequence of
Bacillus PB92 serine protease. Promoters (P_1, P_2), ribosome
binding site (rbs) and termination regions (term) of the DNA

15 sequence are also shown. The numbered solid lines represent
the location of the ten oligonucleotides used for sequencing.

      Figure 6A shows the construction of plasmid pE194-
neo.

      Figure 6B shows the construction of plasmid pMAX-

20 4.

      Figure 7A: Digests prepared with HindIII of
chromosomal DNA of the strains PB92, PBT109 and PBT108 were
subjected to electrophoresis on a 0.5% agarose gel, trans-
ferred to nitrocellulose as described by Southern and

25 hybridized with ^{32}P labeled nick-translated pM58 DNA. The
figure shows an autoradiograph.

      Figures 7B and 7C illustrate the integration events
occurring in case of homologous (B) recombination and
illegitimate (C) recombination between pMAX-4 and the Bacillus

30 PB92 chromosome.

      Figure 8 shows the construction of integration
vector pElatB.

      Figure 9A illustrates the integration of plasmid
pElatB into the chromosome of B. licheniformis strain T9

35 resulting in B. licheniformis strain TB13.

      Figure 9B illustrates the chromosomal recombination
of the B. licheniformis strains TB13 and T5 upon protoplast
fusion of these strains, resulting in B. licheniformis strain
T13F.

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Figure 10 shows a chromosomal analysis of nine different colonies isolated from a fermentation of strain T13F as described in Example 11. Isolated chromosomal DNA was digested with EcoRI separated on 0.8% agarose gels and blotted

5 onto nitrocellulose. Hybridization of the blot was performed with 32P-labeled nick-translated pElatB DNA. The figure shows an autodiagram. The upper arrow indicates the position where an EcoRI DNA fragment of about 15kb migrates which contains the entire pElatB sequence that was integrated into the

10 chromosome on a location not adjacent to the original alpha-amylase gene, as depicted for strain TB13 in Figure 9A. The lower arrow indicates the position where an EcoRI DNA fragment of about 33kb migrates which contains the entire alpha-amylase gene originally present in B. licheniformis strain T5 (see

15 also Figure 9B). The following DNA samples were analyzed:

lane 1: Bacillus licheniformis T5 DNA
lane 2: Bacillus licheniformis TB13 DNA
lane 3: Bacillus licheniformis T390 DNA

20   lane 4: DNA from a neomycin-sensitive derivative of Bacillus licheniformis T390, isolated after fermentation, as described in Example 12.
lane 5: Bacillus licheniformis T13F DNA.
lanes 6-14: DNA from 9 different colonies isolated

25   from a fermentation of strain T13F as described in Example 12.

DESCRIPTION OF THE SPECIFIC EMBODIMENTS

30   In accordance with the present invention, prokaryotic cells, and methods for their preparation, are provided in which two or more copies of a DNA sequence are stably integrated into the chromosome. A host cell comprising a DNA sequence encoding a polypeptide of interest is transformed

35 with a DNA construct comprising said DNA sequence. Transformed cells in which the integrated DNA sequences are separated by endogenous chromosomal sequences from the gene to be amplified are then selected for. The endogenous intervening sequences

are generally vital to the host cell. Loss of amplified
sequences by homologous recombination will be lethal to the
host cell. Thus, there will be selection pressure for cells
carrying the amplified sequences without the necessity for
5 using antibiotics or like selection means. Integration may be
achieved either by homologous recombination or by illegitimate
recombination. Techniques which can be used to obtain the
desired cells are as shown in Figures 2A and 2B, respectively.
       When homologous recombination is used, several
10 stretches of DNA sequences can be present in the vector
molecules which are homologous to the host cell chromosome,
especially when one or more copies of the gene to be amplified
have already been introduced into the host cell. The vector
molecule thus can include a DNA sequence of interest; a target
15 DNA sequence; and a marker DNA sequence.
       Care has to be taken that only the desired
recombined chromosomal arrangements are selected for. This can
be achieved by using linear DNA molecules for recombination.
The circular vector molecule to be integrated is cut with a
20 restriction enzyme in the region homologous to the target
sequence. In this way recombination and integration at this
specific site can occur preferentially. In addition to being
present in the vector molecule, the DNA sequence of interest
can also be present in the host cell chromosome. The DNA
25 sequence may be a DNA sequence encoding any structural gene
which it is desired to amplify. The DNA sequence may be
endogenous to the host organism, or may have been inserted
into the host chromosome in a previous transformation step.
       Target sequences for non-tandem gene amplification
30 will preferably be chosen from among non-essential genes, for
example in the case of Bacilli as host organisms, the genes
encoding extracellular enzymes or genes involved in
sporulation can be used as target sequences. Integration of
DNA sequences in these genes will generally inactivate the
35 gene. Loss of expression of the gene can then be monitored and
used for the selection of the desired recombinant strains.
       When illegitimate recombination is used for
chromosomal gene amplifiation as depicted in Figures 1D and

2A, conditions for integration and selection are preferred in
which homologous recombination does not predominate over
illegitimate recombination. A preferred means of avoiding
homologous recombination is to transform first and second host
5 cells which lack the structural gene of interest with a vector
comprising a DNA sequence encoding a polypeptide of interest,
and a marker gene. First and second host cells in which the
DNA sequence is present at different locations can then be
selected and combined under fusing conditions to yield a
10 transformed cell with at least two copies of the DNA sequence
encoding the structural gene of interest at scattered
locations in the second host genome. For ease of selection the
first host can be killed prior to fusion.

        The gene(s) of interest may be any prokaryotic or
15 eukaryotic gene. These genes may include bacterial genes,
unicellular microorganism genes, mammalian genes, or the like.
The structural genes may be prepared in a variety of ways,
including synthesis, isolation from genomic DNA, preparation
from cDNA, or combinations thereof. The various techniques of
20 manipulation of the genes are well-known, and include
restriction, digestion, resection, ligation, in vitro muta-
genesis, primer repair, employing linkers and adapters, and
the like. Thus, DNA sequences obtained from a host may be
manipulated in a variety of ways, depending upon the require-
25 ments of the DNA construction. See Maniatis et al., Molecular
Cloning, Cold Spring Harbor Laboratory, Cold Spring Harbor,
NY, 1982.

        The structural genes may express a variety of poly-
peptides or proteins, such as enzymes, hormones, lymphokines,
30 surface proteins, blood proteins, structural proteins, immuno-
globulins, or the like, from mammals, unicellular microorga-
nisms, e.g., bacteria, fungi, such as yeast, or filamentous
fungi, algae, protozoa, etc., plants or other DNA source. Of
particular interest are enzymes, more particularly proteases
35 and amylases. Illustrative of such enzymes are serine and non-
serine proteases, including high alkaline serine proteases,
alpha- and bêta-amylase, and the like. A preferred source for
a serine protease is Bacillus novo species PB92, and for an

alpha-amylase is B. licheniformis strain T5, as well as
mutants and variants of these strains.

The gene that forms part of the suitable vector can
be obtained by methods generally known in the art. Generally,
5 the method comprises preparing a genomic library from the
organism expressing a high alkaline protease. The genomic
library is conveniently prepared for example by ligating DNA
fragments of the donor strain into a suitable vector.

By the term "suitable vector" is meant a DNA
10 construct comprising a structural gene encoding a protein of
polypetide of interest. The structural gene is joined in
proper orientation to control regions such as a promoter
sequence, a sequence forming the ribosome binding site and
sequences controlling termination of transcription and
15 translation of the structural gene, which control regions are
functional in the host cell. Where the host cell has
transformation and integration frequencies which are too low
to permit direct selection for integration without inter-
mediate isolation of plasmid containing cells, such as
20 industrial Bacillus strains, the vector can additionally
comprise an origin of replication that is capable of
replicating autonomously in the host cell.

Where the gene is obtained from a donor cell which
has transcriptional and translational initiation and
25 termination regulatory signals which are recognized by the
host prokaryotic cell strain, it will usually be convenient to
maintain the original regulatory sequences of the structural
gene. In addition, the transcriptional initiation region may
provide for constitutive or inducible expression, so that in
30 appropriate situations, the host may be grown to high density
before high levels of expression of the structural genes of
interest are obtained.

Where the structural gene is from a source whose
regulatory signals are not recognized by the host cell, it
35 will be necessary to obtain regulatory regions recognized by
the host cell and to insert the structural gene between the
initiation and termination regulatory signals. In some
instances the exogenous structural gene with its own stop

codon(s) may be inserted in reading frame behind the N-terminus codons of an endogenous structural gene which retains its natural regulatory signals.

It is desirable that the expression product be
5 secreted. Where the expression product is naturally secreted and the leader signals and processing signal(s) are recognized by the host cell, this will entail no difficulty. However, where the product is not secreted because the host cell does not recognize the secretory leader signals and/or processing
10 signal(s), or the signals are not functional to a satisfactory degree in the host cell, then it may be necessary to isolate or synthesize DNA sequences coding for the secretory leader signals and processing signal(s) of a host cell polypeptide and join them in proper reading frame to the 5'-end of the
15 structural gene.

The vector may additionally include a marker gene conferring resistance to an antibiotic to which the host strain is sensitive. The marker gene, when used in chromosomal integration of the vector, has to fulfill the demand that
20 survival selection is possible even if only one or a few copies of the marker gene are present in the host strain. By marker is intended a structural gene capable of expression in a host, which provides for survival selection. By "survivial selection" is intended imparting prototrophy to an auxotrophic
25 host, biocide or viral resistance. For prototrophy, various genes may be employed, such as leu, his, trp, or the like. For biocide resistance this may include resistance to antibiotics, e.g., neo, cam, tet, tun, kan, or the like. Other markers include resistance to heavy metals, immunity, and the like.
30 The various DNA sequences may be derived from diverse sources and joined together to provide for a vector which includes one or more convenient, preferably unique, restriction sites to allow for insertion or substitution of the structural genes at such sites or in place of lost fragments to provide the
35 plasmid construct.

Selection for chromosomal integration may be aided by using a plasmid with an origin replication having a mutation which makes its functioning temperature-sensitive in

- 13 -

the host cell. See, for example, Ehrlich, Proc. Natl. Acad.
Sci. USA 75 (1978) 1433.

Once the plasmid construct has been prepared, it
may now be cloned in an appropriate cloning host. Any host may
5 be used which is convenient, is readily transformable, and
allows for replication of the plasmid construct and transfer
to the host cell. A large number of strains are available
which have a high efficiency of transformation and are usually
auxotrophic and/or antibiotic sensitive. Where the host cell
10 is an industrial Bacillus strain, the use of the same organism
as the host cell for cloning of the plasmid construct has many
advantages in that it permits the use of a single replication
system as well as the same marker for survival selection in
both the cloning host and the host strain. See, for example,
15 European application EP-A-134048, which disclosure is incorp-
orated herein by reference.

The plasmid construct may be introduced into the
cloning host in accordance with conventional techniques, such
as transformation, employing calcium precipitated DNA, con-
20 jugation, or other convenient technique. The cloning host may
then be grown in an appropriate nutrient medium, under
selective conditions to select for a host containing the
plasmid construct. For auxotrophic hosts, the nutrient medium
is deficient in the required nutrient, while for biocide
25 resistance, e.g., antibiotic resistance, a cytotoxic amount of
the biocide(s) is employed in the nutrient medium.

Various host cells may be employed. These include
E. coli, Bacillus strains, expecially Bacillus subtilis,
Pseudomonas, and Streptomyces. In choosing a host cell,
30 various factors are taken into account, including factors
which can affect expression of the gene to be amplified and
production of the desired product. Thus it is desirable to use
a host cell in which there is recognition of regulatory
signals; ease of secretion; reduced degradation of the desired
35 product, etc. A preferred host cell already produces the
polypeptide of interest, and may be either a wild type
organism or a mutant organism. The host cell can also be a
mutant of an organism which produces the polypeptide of

interest which itself, however, is non-producer. Where the
polypeptide of interest is a protease or an amylase, preferred
strains include <u>Bacillus</u> novo species PB92 and <u>Bacillus</u>
<u>licheniformis</u> strain T5, respectively, as well as mutants and
5 variants of these strains.

In addition, industrial strains may be employed
which have the desired traits of an industrial strain.
Examples of strains which may be employed include strains used
for the industrial production of enzymes such as: <u>B. licheni-</u>
10 <u>formis</u>, <u>B. amyloliquefaciens</u> and alkalophilic Bacilli. The
industrial strains are chosen from organisms which may be
isolated in the soil or available from depositories or other
sources or obtained by modification of such strains. The
industrial strains are highly robust and stable. Furthermore,
15 said strains are resistant to phage infection and to genetic
exchange, that is introduction of DNA by conventional
transformation procedures. The conventional industrial strains
are also prototrophic, in order to avoid adding expensive
amino acids to the nutrient medium. Other characteristics of
20 industrial strains are their high productivity until the end
of the fermentation, which can be as long as a week, stable
cell concentration upon exhaustion of the broth, and high
productivity, usually at least 5 g/l (0.5% w/v) of a specific
secreted protein.

25   Transformants can be obtained having genes either
in tandem arrangement or scattered in the chromosome. In
general, it is possible to select transformants containing
scattered genes from mixtures of the two types of
transformants mentioned, by isolating chromosomal DNA of each
30 individual transformant, subsequently analyzing said DNA with
respect to the relative locations of said genes by, for
example, the method of Southern, J. Mol. Biol. <u>98</u> (1975) 503-
517 or other means known to those skilled in the art, thereby
identifying scattered integration of said genes.

35   Means for obtaining scattered gene transformants
avoiding tandem integration include, using double reciprocal
recombination as illustrated in the Figure 1B and 2B, the use
of linearized DNA constructs comprising the DNA sequence to be

amplified, a marker gene and target sequences for recombin-
ation.

Furthermore, specific means for obtaining scattered
gene transformants include the use of illegitimate recombin-
5 ation as illustrated in Figure 2A, in which isolation of
tandem transformants can be avoided by selection, using
differential expression of a marker gene, for example a gene
encoding antibiotic resistance, where sensitivity to the anti-
biotic is different in strains with tandem integration of the
10 gene as opposed to non-tandem integration. Generally, the
length of the intervening endogenous DNA sequences will be
less than 10 kbp.

Additionally, means for obtaining scattered gene
transformants avoiding tandem duplication include the use of
15 killed protoplasts of a homologous donor strain carrying a DNA
construct comprising the structural gene and a marker gene,
the structural gene being integrated in the chromosomal at a
different location with respect to the acceptor strain.

Transformation of the host cells preferably
20 involves the use of protoplasts prepared from the host strain.
Protoplasts generally are prepared from the cells in
accordance with conventional ways, e.g. lysozyme or zymolyase
treatment, and the protoplasts carefully suspended in an
appropriate medium having proper osmolalities for maintaining
25 the integrity of the protoplast. For industrial Bacillus
strains, methods for preparing protoplasts are described in
EP-A-0134048, which disclosure is incorporated herein by
reference. Where the host strain is an alkalophilic Bacillus
strain, protoplasts may conveniently be prepared at alkaline
30 pH, preferably about pH 8.0. This procedure is disclosed in
European Application No. EP-A-87200358.7, which disclosure is
incorporated herein by reference.

The host cell can be transformed by combining the
plasmid construct or a cloning host protoplast with the host
35 cell protoplast in the presence of an appropriate fusogen. Any
fusogen may be employed which provides the desired degree of
efficiency, for the most part polyethylene glycol is found to
provide high efficiency of fusion with great convenience.

After a short time, the fusogen mixture is replaced with an
appropriate nutrient medium and cells regenerated in a
selective medium, conveniently by plating on an agar plate.

Transformants obtained by combining a host cell
5 with a suitable DNA construct can contain said DNA construct
or part thereof either directly as an integral part of their
chromosome or as free vector molecules when the DNA constructs
contain an origin of replication functional in said host cell.

A means of selecting for transformants wherein the
10 DNA construct is integrated into the chromosome is to use a
plasmid containing a temperature-sensitive origin of
replication. Transformants are grown in a selective medium at
the permissive temperature, then shifted to a non-permissive
temperature. Colonies expressing the marker gene at the non-
15 permissive temperature are then isolated and cultured in
selective medium at the permissive temperature. Plasmid
absence can be verified, for example, by isolating total DNA
from the colonies and electrophoresising on an agarose gel or
by demonstrating lack of ability of the transformants to
20 transform competent cells. Determination of the way in which
integration into the chromosome has taken place can be
analysis of the chromosomal DNA by, for example, the method of
Southern, J. Mol. Biol. 98 (1975) 503-517 or other means known
to those skilled in the art.

25   When there is a differential sensitivity to the
selective agent between transformants containing additional
copies of the marker gene in a tandem array as compared to
those in which the marker gene is incorporated at scattered
locations in the host genome, transformants can conveniently
30 be grown in medium containing the appropriate concentration of
selective agent to select for transformants with non-tandem
integration.

Another means of obtaining transf rmants with
scattered integration of copies of the DNA sequence of
35 interest is to use a protoplast prepared from a homologous
donor cell, containing at least one copy of the DNA sequence
of interest at a location on its chromosome different from
that of the recipient host cell. The homologous donor cell can

be prepared, for example, by transforming a cell which does
not contain the structural gene of interest with a vector
comprising the structural gene. Integration of the DNA
sequence into the donor cell chromosome can be facilitated by
5 using a plasmid containing a temperature-sensitive origin of
replication and growing transformants under selective
conditions first at the permissive temperature and then at the
non-permissive temperature as described above, then isolating
colonies expressing the marker gene.

10   Following verification of the absence of plasmid
DNA, the chromosomal DNA can be isolated and analyzed
according to the method of Southern, supra, by hybridizing
with a probe labeled with, for example, ^{32}P or biotinylated
nucleotides. The probe may be cDNA encoding the polypeptide of
15 interest or fragments thereof as well as DNA constructs or
fragments thereof comprising the DNA sequence of interest, for
example a vector. Transformants containing the gene of
interest at an alternate location as compared to that of the
gene donor strain can then be used as an homologous donor
20 cell. The recipient strain host is preferably the same as the
strain used as the source of the DNA sequence of interest, or
a strain in which the DNA sequence of interest is located at a
different region of the chromosome than in the transformed
donor cell.

25   To aid in selection, the donor cell preferably is
killed with a cytotixic agent prior to or during protoplast
formation. Various agent may be employed to kill the donor
cell, including antibiotics, but iodoacetamide is found to be
convenient, efficient, and does not interfere with the
30 subsequent fusion. When dead cloning host protoplasts are
used, the ratio of dead protoplast to the acceptor strain host
will be preferably at least about 1:1 and an excess of the
dead protoplast may be employed.

          Following fusion of the dead donor cell protoplast
35 and the recipient host cell protoplast, transformants can be
selected by means of the marker gene. DNA can then be isolated
and analyzed as described above to identify transformants in
which more than one copy of the gene of interest has been

- 18 -

incorporated into the genome and are separated by endogenous
chromosomal sequences.

Scattered two-gene transformants are then screened
in appropriate ways for detection of increased expression of
5 the polypeptide of interest. Various techniques may be
employed, particularly where enzymes are involved which have
well established methods of detection. Alternatively, where
enzymes are not involved or there is no available detection
system, bioassays, antibodies, or DNA or RNA hybridization can
10 be employed for screening the clones to determine the presence
of the plasmid construct and expression of the structural gene
of interest.

The host cell containing the chromosomally
integrated plasmid constructs or fragments thereof is then
15 grown in a nutrient medium under conventional fermenting
conditions. The fermenting may be continued until the broth is
exhausted. Where the product has been secreted, the product
may be isolated from the broth by conventional techniques,
e.g., extraction, chromatography, electrophoresis, or the
20 like. Where the product is retained in the cytoplasm, the
cells may be harvested by centrifugation, filtration, etc.,
lysed by mechanical shearing detergent, lysozome, or other
techniques and the product isolated as described previously.
By employing the subject method, stable integration of at
25 least two copies of a DNA sequence can be achieved as a means
of gene amplification.

The following examples are offered by way of
illustration and nor by way of limitation.

30

- 19 -

EXPERIMENTAL

EXAMPLE 1

5   Preparation of a Genomic DNA Library from
       Alkalophilic Bacillus novo sp. PB92 and
       Isolation of the Serine Protease Gene

       Chromosomal DNA isolated from Bacillus novo sp.
10 PB92 (deposited under No. OR-60 with Laboratorium voor
   Microbiologie, Technical University of Delft, the Netherlands,
   see U.S. Patent No. Re. 30,602) according to the procedure
   described by Saito-Miuva, Biochim. Biophys. Acta 72 (1963)
   619-632, was partially digested with the restriction enzyme
15 Sau3A and ligated into the BamHI site of plasmid pUB110
   (Gryczan et al., J. Bacteriol. 134 (1978) 318-329). pUB110
   plasmid DNA was prepared as described by Birnboim and Doly
   (Nucl. Acids Res. 7 (1979) 1513-1523).
       The ligation mixture was transformed into B.
20 subtilis 1A40 (Bacillus Genetic Stock Centre) according to the
   method of Spizizen et al., J. Bacteriol. 81 (1961) 741-746,
   using 0.6-1 μg DNA per ml of competent cells. Cells from the
   transformation mixture were plated on minimal plates
   containing: 2.8% K_2HPO_4, 1.2% KH_2PO_4, 0.4% $(NH_4)_2SO_4$, 0.2%
25 tri-Na-citrate.$2H_2O$, 0.04% $MgSO_4.7H_2O$, 0.00005% $MnSO_4.4H_2O$,
   0.4% L-glutamic acid, 0.5% glucose, 0.02% casamino acids, 50
   μg/ml trypthophan, 20 μg/ml methionine, 20 μg/ml lysine, 20
   μg/ml neomycin, 0.4% casein and 1.5% agar. After overnight
   incubation of the plates at 37°C one out of 50,000 neomycin
30 resistant colonies showed increased protease production, as
   determined by increased precipitation of a halo of casein
   cleavage products around the colony in the agar plate. Plasmid
   DNA was isolated from this colony according to the method
   described by Birnboim and Doly, Nucleic Acids Res. 7 (1979)
35 1513-1523, and named pM58.

EXAMPLE 2

Expression of the PB92 Serine Protease Gene

5   Bacillus subtilis 1A40 containing pM58 was grown in
minimal medium (Spizizen et al., Proc. Natl. Acad. Sci. USA 44
(1958) 1072-1078) to which had been added 0.02% casamino acids
50 µg/ml trypthophan, 20 µg/ml methionine, 20 µg/ml lysine and
20 µg/ml neomycin. After 24 hours, the culture was centrifuged
10 and the supernatant assayed for protease activity using di-
methyl casein as substrate (Lin et al., J. Biol. Chem. 244
(1969) 789-793. A culture of B. subtilis 1A40 containing the
plasmid pUB110 used as a control showed less than 1/60 of the
protease activity shown by the pM58 transformed culture.
15 Protease activity was completely inhibited by treatment with
1 mM phenylsulfonyl fluoride (PMSF), but not by treatment with
20 mM EDTA.

        Aliquots of the above described supernatants were
analyzed on protein gel according to the method of Laemmli,
20 Nature 227 (1970) 680. Samples for analysis on these gels were
prepared by treatment of the supernatants with 5% trichloro-
acetic acid (TCA). Following centrifugation of the sample the
pellet of precipitated protein was washed twice with acetone
then dissolved in 40 µl sample buffer (0.5 M Tris/HCl pH 7.5,
25 10% v/v 2-mercaptoethanol, 50% v/v glycerol and 0.05% Bromo-
phenol Blue) by boiling for 10 minutes. After electrophoresis,
the gels were stained using Coomassie Brilliant Blue. Culture
supernatant samples were then analyzed by electrophoresis.
Three different B. subtilis 1A40 strains were used: a strain
30 containing pUB110; or pM58; or no plasmid; and Bacillus PB92
protease as a control. After electrophoresis, the gels were
stained using Coomassie Brilliant Blue and destained. The
sample from B. subtilis strain 1A40 containing pM58 contained
a 31 kD protein, which comigrates with Bacillus PB92 protease.
35 This protein was not detected on the control lane of strain B.
subtilis 1A40 containing pUB110.

        All serine proteases have similar molecular
weights. The cloned serine protease of Bacillus PB92 therefore

was differentiated from known serine proteases (B. subtilis
subtilisin, Carlsberg subtilisin), by transformation of pM58
and pUB110 to the protease negative B. subtilis strain DB104
(R. Doi, J. Bacteriol. 160 (1984) 442-444) and analysis of the
5 extracellular proteases produced. The obtained transformants
were grown in minimal medium (Spizizen et al., supra)
containing 0.02% casamino acids, 50 µg/ml histidine and 20
µg/ml neomycin. After 24 hours, samples were taken, centrifug-
ed and without pretreatment analysed on histidine/MOPS gels
10 containing 75 mM KOH, 40 mM histidine, 100 mM MOPS (3-(N-
morpholino)-propanesulfonic acid), pH 7.5 and 5% polyacryl-
amide. Electrophoresis buffer contained 40mM histidine, 100 mM
MOPS, pH 6.6. Samples were run in the direction of the
cathode. Protease bands were detected with Agfa Pan 100
15 Professional films (Zuidweg et al., Biotechnol. and Bioengin.
14 (1972) 685-714). These results are shown in Figure 3. As
shown in Figure 4, pM58 harbours the gene encoding Bacillus
PB92 protease.

20   EXAMPLE 3

Sequencing of the Bacillus PB92
Serine Protease Gene

25   The entire sequence of a BalI-HpaI fragment of pM58
was determined by the method of Sanger, Proc. Natl. Acad. Sci.
USA 74 (1977) 6463. Restriction fragments of pM58 (see Figure
4) were cloned in phage M13 vectors mp10, mp11 and mp18
(Messing et al., Nucleic Acids Res. 9 (1981) 309-321. Insert-
30 ions of pM58 fragments were screened by plaque hybridization.
After sequencing, ten oligonucleotides located at regular
distances on the gene were made and sequencing was repeated,
confirming the sequence shown in Figure 5.

35

EXAMPLE 4

Construction of Serine Protease
Containing Plasmid pMAX-4

5

     To construct plasmid pUCN710 (Figure 6A) pUB110 was
digested with TaqI and PvuII. The fragment containing the gene
conferring neomycin resistance was purified on low melting
agarose and made blunt with Klenow polymerase and NTP's
10 (Maniatis, Molecular Cloning: A Laboratory Manual. Cold Spring
Harbor 1982). Plasmid pUC7 (Vieira et al., Gene 19 (1982) 259-
268) was linearized with SalI and made blunt as described
above. Both fragments were ligated with T4 ligase (Maniatis)
and transformed to E. coli JM103. Selection took place on 2xTY
15 plates (1.6% w/v Bacto-trypton, 1% w/v yeast extract, 0.5%
NaCl) containing 50 μg/ml ampicillin and 10 μg/ml neomycin.
The resulting plasmid, named pUCN710, was digested with BamHI.
The plasmid pE194 (Jordanescu, Plasmid 1 (1978) 468-479) was
digested with BclI. The fragments from both digestions were
20 ligated with T4 ligase and transformed to B. subtilis 1A40.
Selection took place on minimal plates containing 20 ug/ml
neomycin (see Example 1). The plasmid obtained, pE194-neo
(Figure 6A) contains the neomycin gene and a temperature
sensitive origin of replication.
25   Subcloning of the protease gene in integration
vector pE194-neo was performed as follows: pM58 (see Example
1) was digested with HpaI and BalI and BglII. Plasmid pE194-
neo was digested with HpaI. These fragments were ligated with
T4 ligase and transformed to B. subtilis 1A40. Transformants
30 were selected based upon neomycin resistance and an increase
in protease production, as judged by casein cleavage products
precipitation (halo formation, see Example 1). Plasmid pMAX-4
was obtained, the structure of which was confirmed by
restriction enzyme analysis (see Figure 6B).

35

- 23 -

EXAMPLE 5

Protoplast Transformation of
Bacillus Strain PB92 by pMAX-4

5   Bacillus strain PB92 was grown overnight in 100 ml
NBSG-X medium (Thorne et al., J. Bacteriol. 91 (1966) 1012-
1020). The culture was centrifuged for 10 minutes at 4,500 rpm
in a Sorvall model GSA rotor. Protoplasts were prepared by
10 incubating the bacilli for one hour at 37°C in 10 ml Alkalic
Holding Medium (AHM) containing 0.5 M sucrose, 0.02 M MgCl$_2$
and 0.02 M Tris/maleate, pH 8.0, in sterile water to which 0.4
mg/ml lysozyme was added. The protoplasts were pelleted (10
minutes at 4,500 rpm), resuspended in 5 ml AHM$^+$ pH 8.0 buffer
15 (AHM buffer to which 3.5% w/v Bacto Penassay Broth and 0.04%
w/v Albumine Merieux had been added) mixed, then repelleted as
above. After being resuspended in 5.0 ml of alkaline holding
medium, 0.5 ml of this suspension of protoplasts were mixed
with 5 μg of demineralized water containing 1 μg of plasmid
20 DNA and incubated for 2 minutes in the presence of 30% w/v
polyethylene glycol 8,000, pH 8.0. After 1:3 dilution with
AHM$^+$ pH 8.0 medium and centrifugation, the pellet was re-
suspended in a small volume (1 ml) of AHM$^+$ and incubated for
2-3 hours. One hundred microliter aliquots were plated on
25 freshly prepared regeneration plates containing 0.5 M Na
succinate/HCl pH 8.0, 1.5% w/v agar, 0.5% w/v casamino acids,
0.5% w/v yeast extract, 0.031 M phosphate buffer pH 8.0, 0.5%
w/v glucose, 0.02 M MgCl$_2$ and 0.02% w/v Albumine Merieux.
These plates also contained 1000 μg/ml neomycin for selection.
30 After incubation at 37°C for at least 72 hrs, the colonies
were replica-plated onto heart infusion agar plates containing
20 μg/ml neomycin.

EXAMPLE 6

35

Integration of pMAX-4 in the
Bacillus Strain PB92 Chromosome

- 24 -

A transformant of <u>Bacillus</u> PB92, containing plasmid pMAX-4, was incubated in Tryptone Soya Broth (TSB) containing either 1 μg/ml or 20 μg/ml neomycin for 24 hrs at 37°C. Two ml portions of the cell suspensions were then diluted in 100 ml

5 of TSB containing 1 μg/ml or 20 μg/ml neomycin, respectively, and incubated for 24 hrs at 50°C. After 24 hrs 5 ml samples of both cultures were diluted again, as described above, and incubated for 24 hrs at 50°C, again in the presence of 1 μg/ml, or 20 μg/ml neomycin, respectively. The last procedure was

10 repeated once more. The cell suspensions were then diluted 100-fold and plated on Heart Infusion (HI) agar plates containing 1 μg/ml neomycin for the samples from the flasks containing 1 μg/ml neomycin, and 20 μg/ml neomycin for the samples from the flasks containing 20 μg/ml neomycin. The

15 plates were incubated for 16 hrs at 50°C. Neomycin-resistant colonies were isolated and cultured in 10 ml TSB medium containing 1 μg/ml neomycin for 16 h at 37°C. From these cultures total DNA was isolated (Holmes <u>et al.</u>, Anal. Biochem. <u>114</u> (1981) 193-197. Plasmid absence was verified by DNA

20 electrophoresis on agarose gel. Absence of plasmid DNA from samples in which plasmid DNA was not detectable was confirmed by transformation of total DNA to <u>B</u>. <u>subtilis</u> 1A40. Samples lacking the ability to transform <u>B</u>. <u>subtilis</u> 1A40 were considered plasmid-free.

25   To check whether and in what way integration of pMAX-4 in the chromosome took place, chromosomal DNA was isolated, digested with <u>HindIII</u>, run on 0.5% DNA agarose gels and blotted to nitrocellulose (Southern, J. Mol. Biol. <u>98</u> (1975) 503-517), and hybridized with ^{32}P labeled nick-

30 translated pM58 (Maniatis, 1982). The result of this analysis is shown in Figure 7A.

Selection at 1 μg/ml neomycin resulted in protease genes tandemly located in the chromosome and separated by plasmid sequences (strain PBT109) as a result of homologous

35 recombination (Campbell-type mechanism). In an accumulation of 30 independently isolated integrants, selection was performed at 1 μg/ml neomycin. One integrant was isolated which contained the plasmid pMAX-4 on a random location in the

chromosome as a result of an illegitimate recombination
(strain PBT122). Selection at 20 μg/ml neomycin resulted in a
copy of plasmid pMAX-4 on a random location in the chromosome
as a result of an illegitimate type of recombination. The
5 latter strain was named PBT108. The genetic organization of
the strains PBT109 and 108 are depicted in Figures 7B and 7C,
respectively. Chromosomal analysis showed that integration in
PBT122 and PBT108 occurred on different locations in the
chromosome.

10

EXAMPLE 7

Stability of the Duplicated Protease Genes in
Strains PBT108 and PBT 109

15

One hundred ml of production medium (containing:
1% starch, 4% lactose, 0.87% K_2HPO_4, 0.5% yeast extract, 0.5%
$(NH_4)_2HPO_4$, 0.2% Tri Na citrate.$2H_2O$, 0.05% $MgSO_4.7H_2O$, 0.07%
$CaCl_2$, 0.068% $FeSO_4.7H_2O$ and antifoam 1 ml/l) without neomycin
20 was inoculated with 0.2 ml of an overnight TSB culture (37°C)
of strain PBT108 or PBT109 in 500ml shake flasks. After
incubation for 44 hrs at 37°C under constant aeration the
culture was tested for neomycin-resistant colonies and for
protease activity.

25   Both strains PBT108 and PBT109 were also tested in
Eschweiler fermenters containing the same production medium to
check the effect of upscaling to 10 l. The results of the
fermentation experiments are summarized in the following
Table 1.

30   Table 1

Strain	Relative Production of Protease*	Percent of Neomycin-Resistant Cells After Fermentation
Control (PB92)	100%	−
PBT108	120%	100%
PBT109	115-120%	75-97%

35

- 26 -

* Protease activity was assayed using dimethylcasein as
   substrate as described by Lin et al., J. Biol. Chem. 244
   (1969) 789-793.

5   Analysis of colonies derived from the Eschweiler
fermentation of PBT109 after 2 days of culturing, showed that
3-25% of these colonies produced at the level of a strain
containing only a single protease gene. These same colonies
were found neomycin-sensitive due to excission of the pMAX-4
10 sequence by homologous recombination. However, analysis of the
colonies derived from the strain PBT108 fermentation
experiment showed that these cells were all neomycin-
resistant. One hundred of these neomycin-resistant colonies
were taken at random and individually tested for protease
15 production potential, to determine whether they contained one
or two productive protease genes. All 100 individually tested
colonies produced at the level of a strain containing two
genes, showing that the two randomly integrated protease genes
in PBT108 are stably maintained under the fermentation
20 conditions used.

EXAMPLE 8

Construction of Integration Vector pElatB

25

         Plasmid pGB34, described in EPA 0134048, was
digested with the restriction enzymes BclI, BglI and BglII.
The restriction fragments obtained were blunt-ended with
Klenow polymerase, then were ligated into the HpaI site of
30 pE194-neo (see Example 6). Plasmid pE194-neo DNA was isolated
as described by Birnboim and Doly (Nucl. Acids. Res. 7 (1979)
1513-1523).

         The ligation mixture was transformed into B. sub-
tilis 1-A40, according to the method of Spizizen et al (J.
35 Bacteriol. 81 (1961) 741-746) using 0.5-1 µg DNA per ml of
competent cells. Cells from the transformation mixture were
plated on minimal plates containing 2.8% K_2HPO_4, 1.2% KH_2PO_4,
0.4% $(NH4)2SO_4$, 0.2% Tri Na citrate.$2H_2O$, 0.04% $MgSO4.7H_2O$,

- 27 -

0.00005% MnSO₄.4H₂O, 0.4% glutamic acid, 0.5% glucose, 0.02%
casamino acids, 50 µg/ml tryptophan, 20 µg/ml methionine, 20
µg/ml lysine, 20 µg/ml neomycin, 0.4% casein, 0.5% starch and
1.5% agar.

5   DNA of alpha-amylase producing colonies was
isolated as described by Birnboim and Doly and checked with
restriction enzymes. From one of these transformants plasmid
pElatB, see Figure 8, was isolated.

10   EXAMPLE 9

         Transformation of the Alpha-Amylase Negative Strain
                Bacillus licheniformis T9 with pElatB

15   Transformation of Bacillus licheniformis strain
T9 was carried out as described in EP-A-0253455 with the
exception that the entire procedure was performed at 30°C
instead of 37°C. Selection for transformants was carried out
on minimal plates containing 20 µg/ml neomycin. All trans-
20 formants produced amylase. Restriction enzyme analysis
performed on DNA prepared as described by Birnboim and Doly
showed that the transformants all contained pElatB.

                            EXAMPLE 10

25

                Integration of pElatB into the
                B. licheniformis T9 chromosome

         Bacillus licheniformis strain T9 containing plasmid
30 pElatB, was inoculated in Tryptone Soya Broth (TSB) containing
20 µg/ml neomycin and incubated for 16 hours at 30°C. A 5 ml
portion of the cell suspension was diluted in 100 ml of the
same medium and incubated at 50°C for 24 hours.
         This procedure was repeated once. The cell
35 suspension was then diluted 100-fold and plated on Heart
Infusion Agar plates containing 10 µg/ml neomycin. After 40
hours of incubation at 50°C, neomycin-resistant colonies were
isolated and cultured in 10 ml TSB medium, containing 10 µg/ml

neomycin, for 16 hours at 30°C. Total DNA from these cultures
was isolated (Holmes et al., Anal. Biochem. 114 (1981) 193-
197). The absence of plasmids in these cells was verified by
DNA electrophoresis on agarose gels. Samples in which low
5 molecular weight DNA was virtually absent, were rechecked on
the presence of plasmid DNA by DNA transformation to B.
subtilis 1-A40 (Spizizen et al., 1961). Samples lacking the
ability to transform B. subtilis 1-A40 to neomycin resistance
were considered plasmid minus.

10   To check whether integration of pElatB took place
and how it took place, chromosomal DNA was isolated from the
transformants (Saito-Minwa, Biochem. Biophys. Acta 72 (1963)
619-632), digested with EcoRI fractionated on 0.5% agarose
gels, blotted onto nitro-cellulose (Southern, J. Mol. Biol. 98
15 (1975) 503-517) and hybridized with ^{32}P labeled nick-trans-
lated pGB33 (see EP-A-0134048). The results from this analysis
are shown in Figure 9A. The data show that illegitimate
recombination of pElatB took place resulting in a strain
containing a single amylase gene on a different locus of the
20 genome as compared with the original Bacillus licheniformis T5
amylase strain. The strain obtained containing pElatB was
named TB13.

                        EXAMPLE 11

25

      Construction of Strain T13F Containing Two Amylase
      Genes Separated by Endogenous Chromosomal Sequences

          In order to develop a strain containing two amylase
30 genes separated by endogenous chromosomal DNA sequences, a
fusion experiment was performed between Bacillus licheniformis
strain T5 (the original amylase gene containing amylase
strain, see EP-A-0134048) and strain TB13 (the randomly
integrated, amylase gene containing strain). Protoplast fusion
35 was performed as described in EP-A-0134048, the disclosure of
which is hereby incorporated by reference. Strain TB13 was
killed with iodoacetamide prior to protoplast formation.
Strain T5 (neomycin sensitive) was not killed. Selection for

fusants took place on the regeneration plates containing 10
ug/ml neomycin.

  To check and identify potential fusants, chromo-
somal DNA was isolated, digested with EcoRI, fractionated on
5 0.5% agarose gels, blotted to nitrocellulose filters
(Southern, J. Mol. Biol. <u>98</u> (1975) 503-517) and hybridized
with ^{32}P labeled nick-translated pGB33 (see EP-A-0134048). The
result of this analysis is shown in Figure 9B. One of the
obtained fusants, T13F, contained two amylase genes separated
10 by endogenous chromosomal sequences.

EXAMPLE 12

<u>Stability of the duplicated amylase genes
in strains T390 and T13F</u>

15

  The stability of strain T13F, a strain containing
two chromosomal amylase genes separated by essential chromo-
somal sequences, was compared with that of strain T390, a
20 strain with two chromosomal amylase genes located in a tandem
array. Preparation of strain T390 is disclosed in EP-A-134048
(page 17, Table I), where it was referred to as B.
licheniformis T5 (pGB33). Strains T13F and T390 were tested
under fermentation conditions, nameley 0.2 ml of an overnight
25 TSB culture (37°C) was inoculated in 500 ml shake flasks
containing 100 ml production medium (see Example 7; after
sterilization the pH was adjusted to 6.9 with NaOH) without
neomycin. After incubation for 6 days at 40°C under constant
aeration the culture was tested for neomycin-resistant
30 colonies and amylase activity. The results of the fermentation
experiments are summarized in the following Table 2.

- 30 -

Table 2

Strain	Relative Amylase Activity	Percent of Neomycin-Resistant Cells After Fermentation*
T5	100%	-
TB13	20%	100%
T13F	120%	100%
T390	200%	88%

* More than thousand colonies were tested per strain.

To exclude the possibility of excission of one amylase gene without concomitant loss of the neomycin gene in strain T13F, 20 colonies derived from T13F fermentation were analyzed. Chromosomal DNA from 20 randomly chosen colonies was isolated and characterized by hybridization experiments as described above. The results of 9 of these analyses are shown in Figure 10. All strains tested contained two amylase genes, as demonstrated by the presence of two alpha-amylase genes containing EcoRI fragments in their chromosomal DNA.

In contrast to the genetic stability of strain T13F, strain T390 was found to be unstable upon fermentation resulting in 12% neomycin-sensitive colonies. One of these colonies was analyzed and found to contain only one alpha-amylase gene (Figure 10, lane 4). This shows that randomly integrated amylase genes are more stable than tandemly integrated genes, under fermentation conditions.

It is evident from the above results that a prokaryotic cell may be obtained in which stable gene amplification is achieved by selecting for transformed cells in which non-tandem integration of at least two copies of the structural gene to be amplified has occurred. Integration may occur by homologous recombination or illegitimate recombination.

All publications and patent applications mentioned in this specification are indicative of the level of skill of

those skilled in the art to which this invention pertains. All
publications and patent applications are herein incorporated
by reference to the same extent as if each individual
publication or patent application was specifically and
5 individually indicated to be incorporated by reference.

The invention now being fully described, it will be
apparent to one of ordinary skill in the art that many changes
and modifications can be made thereto without departing from
the spirit or scope of the appended claims.

10

- 32 -

CLAIMS

1. A transformed prokaryotic host cell comprising
at least two copies of a DNA sequence in its chromosome, said
5 DNA sequence encoding a polypeptide of interest, wherein said
copies are separated by endogenous chromosomal DNA sequences.

2. A transformed prokaryotic host cell comprising
at least two copies of a DNA sequence encoding a polypeptide
10 of interest, wherein said copies are separated by endogenous
chromosomal DNA sequences in the genome of said host cell,
said cell being produced by the method comprising:
    combining a recipient host cell comprising at least
one copy of said DNA sequence integrated into its chromosome
15 with (a) a DNA construct, comprising at least one copy of said
DNA sequence and at least one of a marker gene and a
temperature-sensitive origin of replication or (b) a donor
host cell comprising said DNA construct, under transforming
conditions;
20   selecting for a transformant wherein said DNA
construct is integrated into the chromosome of said
transformant; and
    isolating from among said transformants,
transformed prokaryotic host cells comprising at least two
25 copies of said DNA sequence separated by endogenous DNA
sequences.

3. A transformed prokaryotic host cell according to
Claim 1 or 2, wherein said prokaryotic cell is a Bacillus
30 strain.

4. A transformed prokaryotic host cell according to
Claim 3, wherein said Bacillus strain is an alkalophilic
Bacillus strain or a Bacillus licheniformis host strain.
35

5. A transformed prokaryotic host cell according to
Claim 4, wherein said alkalophilic Bacillus strain is Bacillus
novo species PB92 or a mutant or variant thereof.

- 33 -

6. A transformed prokaryotic host cell according to Claim 4, wherein said <u>Bacillus licheniformis</u> host strain is <u>Bacillus licheniformis</u> strain T5 or a mutant or a variant thereof.

7. A transformed prokaryotic host cell according to Claim 1 or 2, wherein said polypeptide of interest is an enzyme.

8. A transformed prokaryotic host cell according to Claim 7, wherein said enzyme is a proteolytic enzyme or an amylolytic enzyme.

9. A transformed prokaryotic host cell according to Claim 8, wherein said proteolytic enzyme is a serine protease.

10. A transformed prokaryotic host cell according to Claim 9, wherein said serine protease comprises substantially the following amino acid sequence:

H_2N-A-Q-S-V-P-W-G-I-S-R-V-Q-A-P-A-A-H-N-R-G-L-T-G-S-G-V-K-V-A-
V-L-D-T-G-I-S-T-H-P-D-L-N-I-R-G-G-A-S-F-V-P-G-E-P-S-T-Q-D-G-N-
G-H-G-T-H-V-A-G-T-I-A-A-L-N-N-S-I-G-V-L-G-V-A-P-N-A-E-L-Y-A-V-
K-V-L-G-A-S-G-S-G-S-V-S-S-I-A-Q-G-L-E-W-A-G-N-N-G-M-H-V-A-N-L-
S-L-G-S-P-S-P-S-A-T-L-E-Q-A-V-N-S-A-T-S-R-G-V-L-V-V-A-A-S-G-N-
S-G-A-G-S-I-S-Y-P-A-R-Y-A-N-A-M-A-V-G-A-T-D-Q-N-N-N-R-A-S-F-S-
Q-Y-G-A-G-L-D-I-V-A-P-G-V-N-V-Q-S-T-Y-P-G-S-T-Y-A-S-L-N-G-T-S-
M-A-T-P-H-V-A-G-A-A-A-L-V-K-Q-K-N-P-S-W-S-N-V-Q-I-R-N-H-L-K-N-
T-A-T-S-L-G-S-T-N-L-Y-G-S-G-L-V-N-A-E-A-A-T-R-COOH.

11. A transformed prokaryotic host cell according to Claim 8, wherein said amylolytic enzyme is alpha-amylase.

12. A transformed prokaryotic host cell according to Claim 1 or 2, wherein the genome of <u>Bacillus</u> novo species PB92 or a mutant or variant thereof comprises said DNA sequence.

13. A transformed prokaryotic host cell according to

Claim 1 or 2, wherein the genome of <u>Bacillus licheniformis</u>
strain T5 or a mutant or a variant thereof comprises said DNA
sequence.

5   14. A method for preparing a transformed prokaryotic

host cell comprising at least two copies of a DNA sequence
encoding a polypeptide of interest, wherein said copies are
separated by endogenous chromosomal sequences in the genome of
10 said host cell, said method comprising:
          combining a recipient host cell comprising at least
one copy of said DNA sequence integrated into its chromosome
with (a) a DNA construct, comprising at least one copy of said
DNA sequence and at least one of a marker gene and a
15 temperature-sensitive origin of replication or (b) a donor
host cell comprising said DNA construct, under transforming
conditions;
          selecting for a transformant wherein said DNA
construct is integrated into the chromosome of said
20 transformant; and
          isolating from among said transformants,
transformed prokaryotic host cells comprising at least two
copies of said DNA sequence separated by endogenous DNA
sequences.
25

          15. A method according to Claim 14, wherein said
selecting comprises:
          growing said transformant comprising a DNA
construct comprising a marker gene in the presence of a
30 biocide to which said marker gene provides resistance; and
          identifying and isolating from said transformants,
plasmid free transformants.

          16. A method according to Claim 14, wherein said
35 selecting comprises:
          growing said transformant comprising a DNA
construct comprising a marker gene and a temperature-sensitive
origin of replication in the presence of a biocide at a non-

permissive temperature; and

selecting from said transformants, plasmid free
transformants.

5   17. A method according to Claim 14, wherein said
isolating comprises:

isolating chromosomal DNA from said transformants;
and

hybridizing said chromosomal DNA with a labeled
10 probe comprising said DNA construct or a fragment thereof
whereby said transformed prokaryotic host cells are selected
by detecting said label.

18. A method according to Claim 14, wherein said
15 donor host cell is obtained by a method comprising:

combining a prokaryotic cell lacking a DNA sequence
encoding said polypeptide of interest with said DNA construct
under fusing conditions;

isolating transformed cells;
20   growing said transformed cells at a non-permissive
temperature; and

identifying and isolating transformed cells wherein
said DNA construct is integrated into a location on the
chromosome other than that in said recipient host cell.

25

19. A method according to any one of Claims 14-18,
wherein said prokaryotic cell is a <u>Bacillus</u>.

20. A method according to Claim 19, wherein said
30 <u>Bacillus</u> is an alkalophilic <u>Bacillus</u> strain or a <u>B. licheni-</u>
<u>formis</u> strain.

21. A method according to Claim 20, wherein said
alkalophilic <u>Bacillus</u> strain is <u>Bacillus</u> novo species PB92 and
35 said <u>B. licheniformis</u> strain is <u>B. licheniformis</u> strain T5.

22. A method according to any one of Claim 14-21,
wherein said polypeptide of interest is an enzyme.

23. A method according to Claim 22, wherein said enzyme is a serine protease or an amylase.

24. A method according to Claim 23, wherein said
5 serine protease has at least 70% homology in nucleotide sequence with a proteolytic enzyme encoding gene from <u>Bacillus</u> novo species PB92.

25. A method according to Claim 23 or 24, wherein
10 said serine protease has substantially the following amino acid sequence:

H₂N-A-Q-S-V-P-W-G-I-S-R-V-Q-A-P-A-A-H-N-R-G-L-T-G-S-G-V-K-V-A-
V-L-D-T-G-I-S-T-H-P-D-L-N-I-R-G-G-A-S-F-V-P-G-E-P-S-T-Q-D-G-N-
15 G-H-G-T-H-V-A-G-T-I-A-A-L-N-N-S-I-G-V-L-G-V-A-P-N-A-E-L-Y-A-V-
K-V-L-G-A-S-G-S-G-S-V-S-S-I-A-Q-G-L-E-W-A-G-N-N-G-M-H-V-A-N-L-
S-L-G-S-P-S-P-S-A-T-L-E-Q-A-V-N-S-A-T-S-R-G-V-L-V-V-A-A-S-G-N-
S-G-A-G-S-I-S-Y-P-A-R-Y-A-N-A-M-A-V-G-A-T-D-Q-N-N-N-R-A-S-F-S-
Q-Y-G-A-G-L-D-I-V-A-P-G-V-N-V-Q-S-T-Y-P-G-S-T-Y-A-S-L-N-G-T-S-
20 M-A-T-P-H-V-A-G-A-A-A-L-V-K-Q-K-N-P-S-W-S-N-V-Q-I-R-N-H-L-K-N-
T-A-T-S-L-G-S-T-N-L-Y-G-S-G-L-V-N-A-E-A-A-T-R-COOH.

26. A method according to any one of Claims 14-25, wherein said DNA construct is pMAX-4 or pElatB.
25

27. A method according to any one of Claims 14-26, wherein said DNA sequence is integrated by illegitimate recombination.

30   28. A method according to any one of Claims 14-26, wherein said DNA sequence is integrated by homologous recombination.

29. A method according to any one of Claims 14-26,
35 wherein said DNA construct further comprises a temperature sensitive origin of replication.

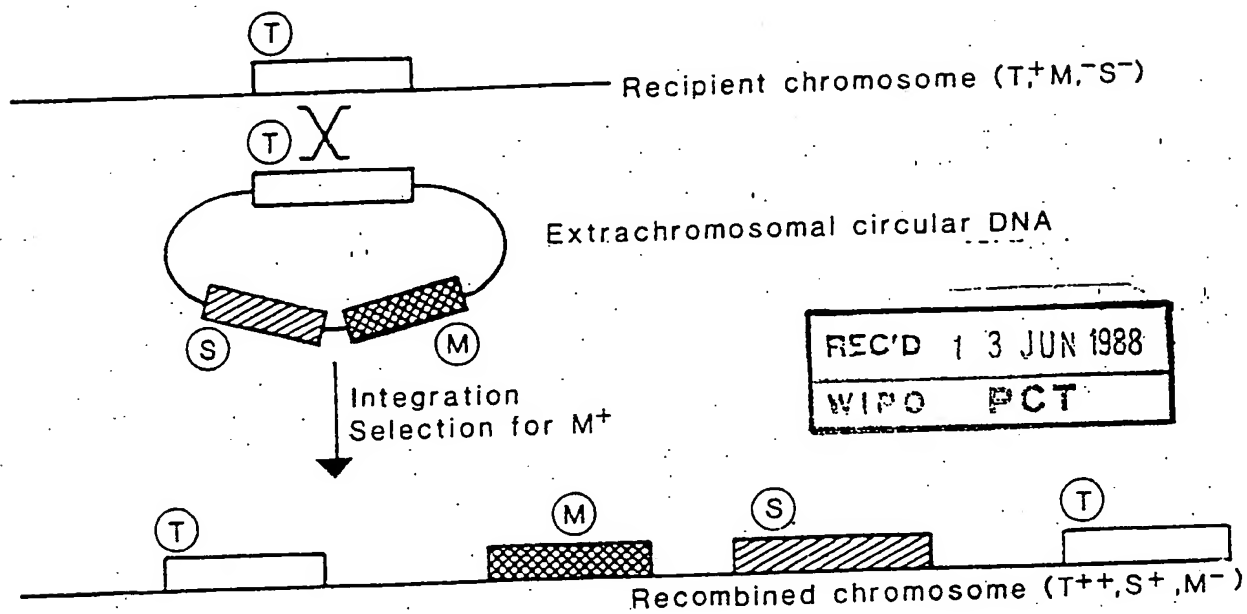30. A method according to of Claim 29, wherein said

temperature sensitive plasmid is derived from plasmid pE194.

    31. <u>Bacillus</u> PBT108, PBT122, or T13F.

5

A. CAMPBELL-TYPE RECOMBINATION
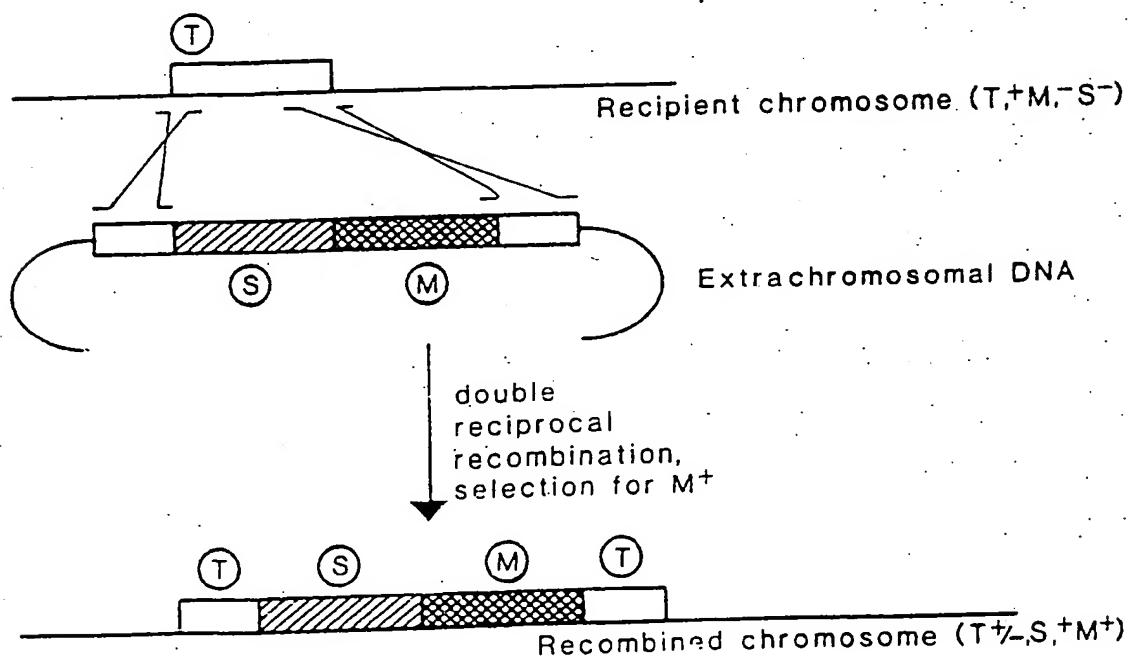

B. DOUBLE RECIPROCAL RECOMBINATION

FIGURE 1

C. TWO-STEP INTEGRATION REPLACEMENT MECHANISM

Recipient chromosone (T,+M,-S-)

Extrachromomal circular DNA

Integration, selection for M+

Intermediate recombined chromosome (T+,S+,M+)

Deletion, selection for M-

Final recombined chromosome (T,+/-S+,M-)
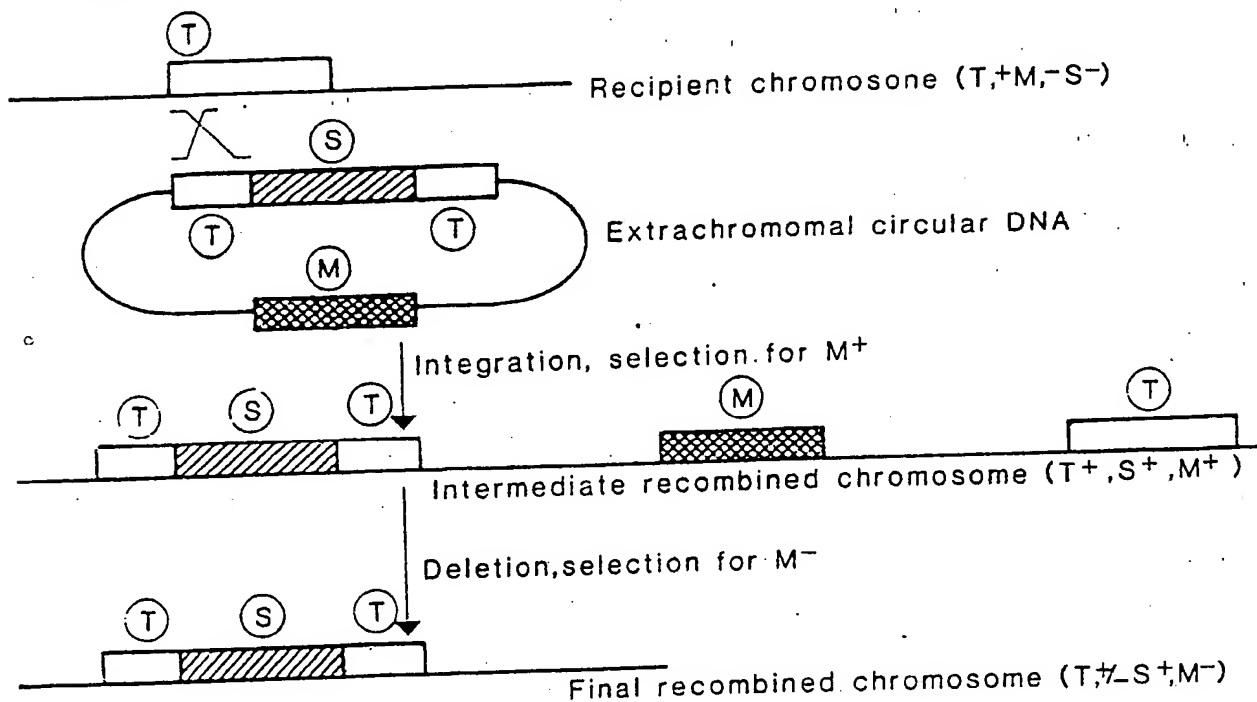
D. INTEGRATION BY ILLEGITIMATE RECOMBINATION

Recipient chromosome (S,-M-)

Extra chromosomal circular DNA

Integration
Selection for M+

Recombined chromosome (S,+M+)

FIGURE 1 (contn'd)

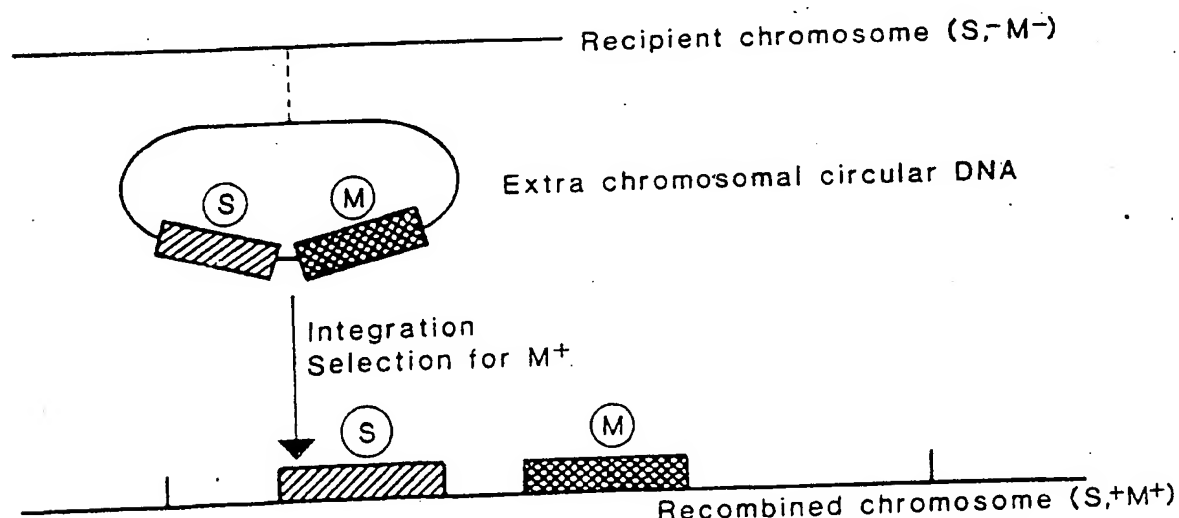A. SPREAD INTEGRATION AND AMPLIFICATION OF DNA SEQUENCES USING ILLEGITIMATE RECOMBINATION

FIGURE 2

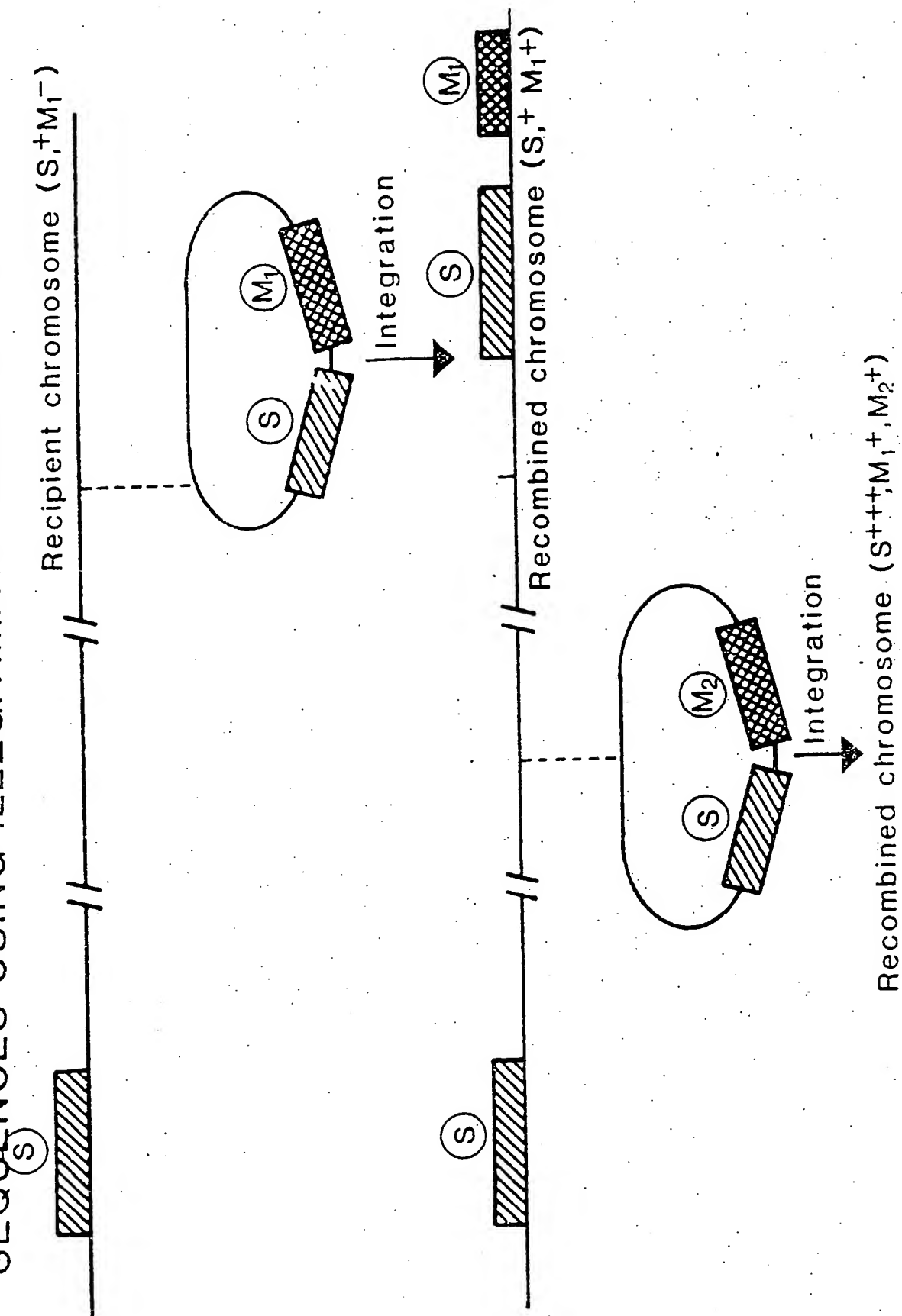B. SPREAD INTEGRATION AND AMPLIFICATION OF DNA
   SEQUENCES USING HOMOLOGOUS RECOMBINATION

FIGURE 2 (contn'd)

FIGURE 3

FIGURE 4

7/18

```
                                                            P1
           20                      40                              60
GATTCTGTTAACTTAACGTTAATATTTGTTTCCCAATAGGCAAATCTTTCTAACTTTGAT


      P2                              RBS              Pre
            80                      100              ┌─120
ACGGTTTAAACTACCAGCTTGGACAAGTTGGGATAAAAATGAGGAGGGAACCGAATGAAG
    ‾‾‾‾‾‾‾‾‾‾‾‾                  ‾‾‾‾‾‾‾‾    ‾‾‾‾‾‾‾
                                                            MetLys

      1.
                   140                   160               180
‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾
AAACCGTTGGGGAAAATTGTCGCAAGCACCGCACTACTCATTTCTGTTGCTTTTAGTTCA
LysProLeuGlyLysIleValAlaSerThrAlaLeuLeuIleSerValAlaPheSerSer


            Pro                              2.
          ┌─200─┐               220               240
          │                              ‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾
TCGATCGCATCGGCTGCTGAAGAAGCAAAAGAAAAATATTTAATTGGCTTTAATGAGCAG
SerIleAlaSerAlaAlaGluGluAlaLysGluLysTyrLeuIleGlyPheAsnGluGln


            260                   280               300
‾‾
GAAGCTGTCAGTGAGTTTGTAGAACAAGTAGAGGCAAATGACGAGGTCGCCATTCTCTCT
GluAlaValSerGluPheValGluGlnValGluAlaAsnAspGluValAlaIleLeuSer


                                   3.
            320               340               360
                        ‾‾‾‾‾‾‾‾‾‾‾‾‾‾‾
GAGGAAGAGGAAGTCGAAATTGAATTGCTTCATGAATTTGAAACGATTCCTGTTTTATCC
GluGluGluGluValGluIleGluLeuLeuHisGluPheGluThrIleProValLeuSer


            380               400               420
GTTGAGTTAAGCCCAGAAGATGTGGACGCGCTTGAACTCGATCCAGCGATTTCTTATATT
ValGluLeuSerProGluAspValAspAlaLeuGluLeuAspProAlaIleSerTyrIle


                     mat     4.
            440       ┌───── 460               480
                     │
GAAGAGGATGCAGAAGTAACGACAATGGCGCAATCAGTGCCATGGGGAATTAGCCGTGTG
GluGluAspAlaGluValThrThrMetAlaGlnSerValProTrpGlyIleSerArgVal
```
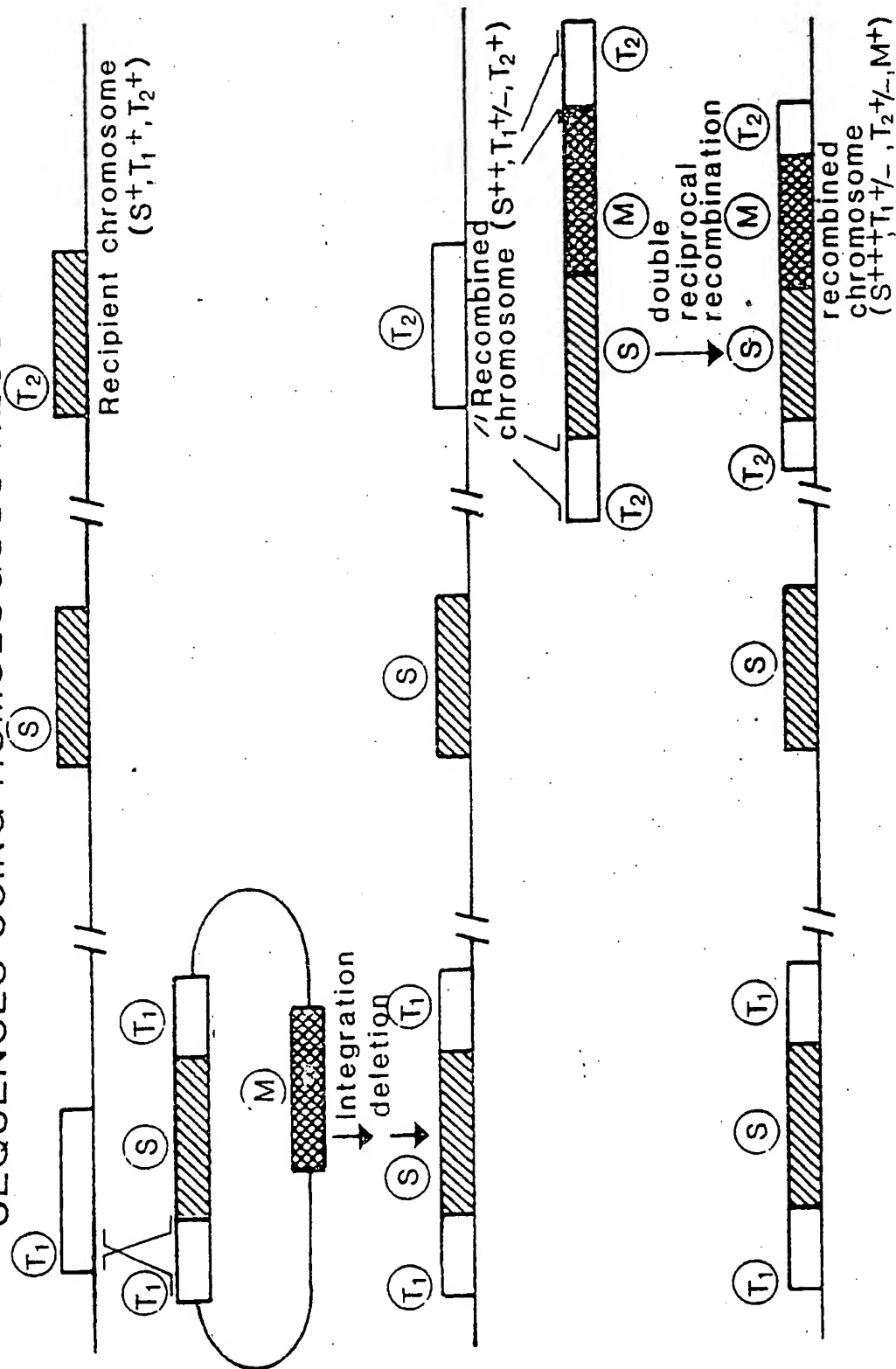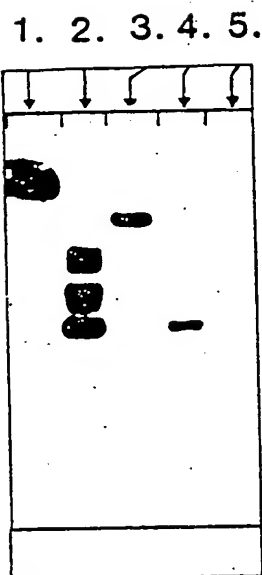
FIGURE 5

```
                    500                520                540
CAAGCCCCAGCTGCCCATAACCGTGGATTGACAGGTTCTGGTGTAAAAGTTGCTGTCCTC
GlnAlaProAlaAlaHisAsnArgGlyLeuThrGlySerGlyValLysValAlaValLeu


                          5.
                    560                580                600
GATACAGGTATTTCCACTCATCCAGACTTAAATATTCGTGGTGGCGCTAGCTTTGTACCA
AspThrGlyIleSerThrHisProAspLeuAsnIleArgGlyGlyAlaSerPheValPro


                    620                640                660
GGGGAACCATCCACTCAAGATGGGAATGGGCATGGCACGCATGTGGCTGGGACGATTGCT
GlyGluProSerThrGlnAspGlyAsnGlyHisGlyThrHisValAlaGlyThrIleAla


              6.
                    680                700                720
GCTTTAAACAATTCGATTGGCGTTCTTGGCGTAGCACCGAACGCGGAACTATACGCTGTT
AlaLeuAsnAsnSerIleGlyValLeuGlyValAlaProAsnAlaGluLeuTyrAlaVal


                    740                760                780
AAAGTATTAGGGGCGAGCGGTTCAGGTTCGGTCAGCTCGATTGCCCAAGGATTGGAATGG
LysValLeuGlyAlaSerGlySerGlySerValSerSerIleAlaGlnGlyLeuGluTrp


              7.
                    800                820                840
GCAGGGAACAATGGCATGCACGTTGCTAATTTGAGTTTAGGAAGCCCTTCGCCAAGTGCC
AlaGlyAsnAsnGlyMetHisValAlaAsnLeuSerLeuGlySerProSerProSerAla


                    860                880                900
ACACTTGAGCAAGCTGTTAATAGCGCGACTTCTAGAGGCGTTCTTGTTGTAGCGGCATCT
ThrLeuGluGlnAlaValAsnSerAlaThrSerArgGlyValLeuValValAlaAlaSer


                    8.
                    920                940                960
GGGAATTCAGGTGCAGGCTCAATCAGCTATCCGGCCCGTTATGCGAACGCAATGGCAGTC
GlyAsnSerGlyAlaGlySerIleSerTyrProAlaArgTyrAlaAsnAlaMetAlaVal
```

FIGURE 5 (contn'd)

980   1000   1020

GGAGCTACTGACCAAAACAACAACCGCGCCAGCTTTTCACAGTATGGCGCAGGGCTTGAC

GlyAlaThrAspGlnAsnAsnAsnArgAlaSerPheSerGlnTyrGlyAlaGlyLeuAsp

9.   1040   1060   1080

ATTGTCGCACCAGGTGTAAACGTGCAGAGCACATACCCAGGTTCAACGTATGCCAGCTTA

IleValAlaProGlyValAsnValGlnSerThrTyrProGlySerThrTyrAlaSerLeu

10.
1100   1120   1140

AACGGTACATCGATGGCTACTCCTCATGTTGCAGGTGCAGCAGCCCTTGTTAAACAAAAG

AsnGlyThrSerMetAlaThrProHisValAlaGlyAlaAlaAlaLeuValLysGlnLys

1160   1180   1200

AACCCATCTTGGTCCAATGTACAAATCCGCAATCATCTAAAGAATACGGCAACGAGCTTG

AsnProSerTrpSerAsnValGlnIleArgAsnHisLeuLysAsnThrAlaThrSerLeu

1220   1240   1260

GGAAGCACGAACTTGTATGGAAGCGGACTTGTCAATGCAGAAGCGGCAACACGCTAATCA

GlySerThrAsnLeuTyrGlySerGlyLeuValAsnAlaGluAlaAlaThrArg

1280   1300   1320

ATAAAAACGCTGTGCTTAAAGGGCACAGCGTTTTTTTTGTGTATGAATCGAAAAAGAGAAC
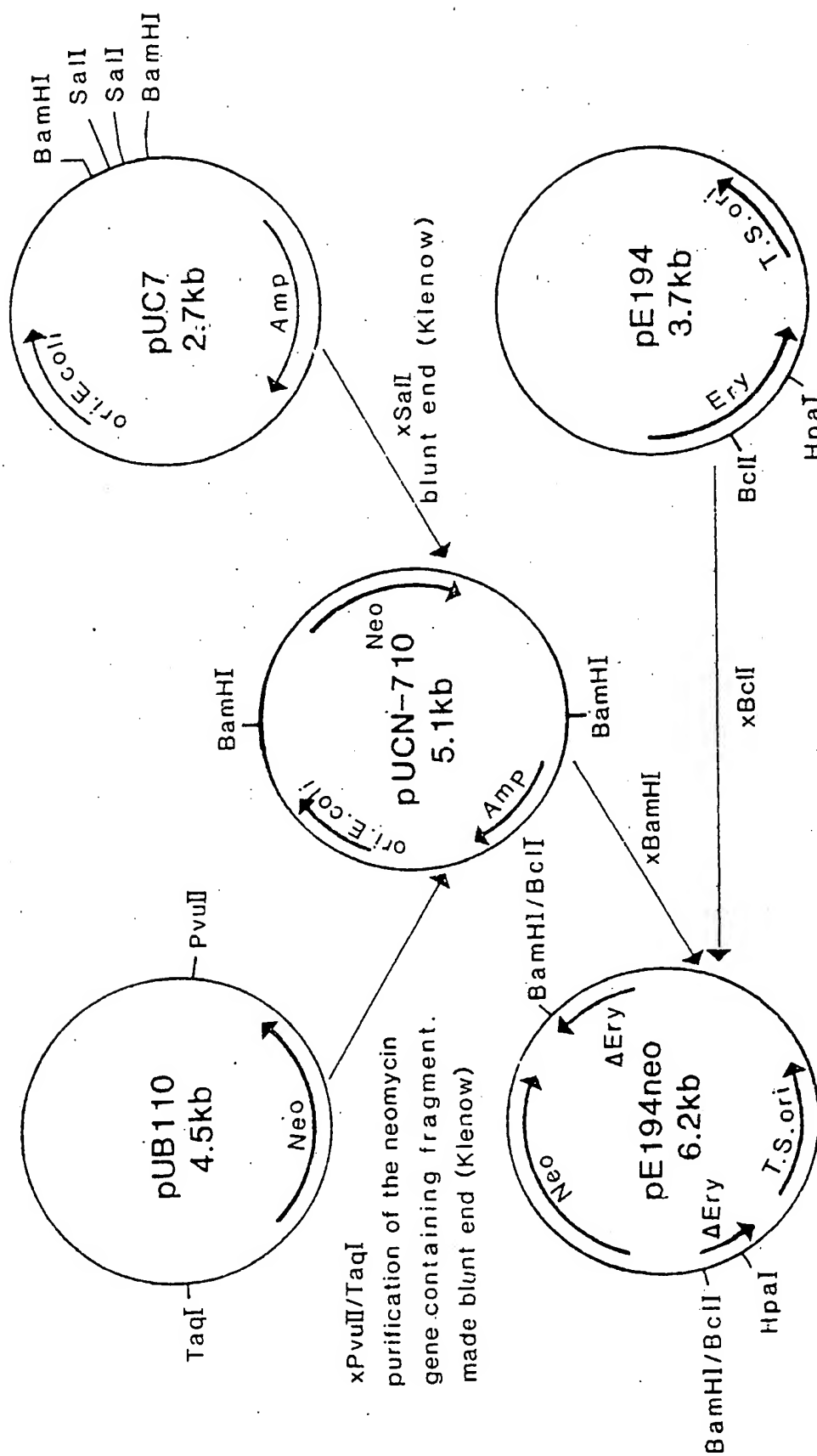
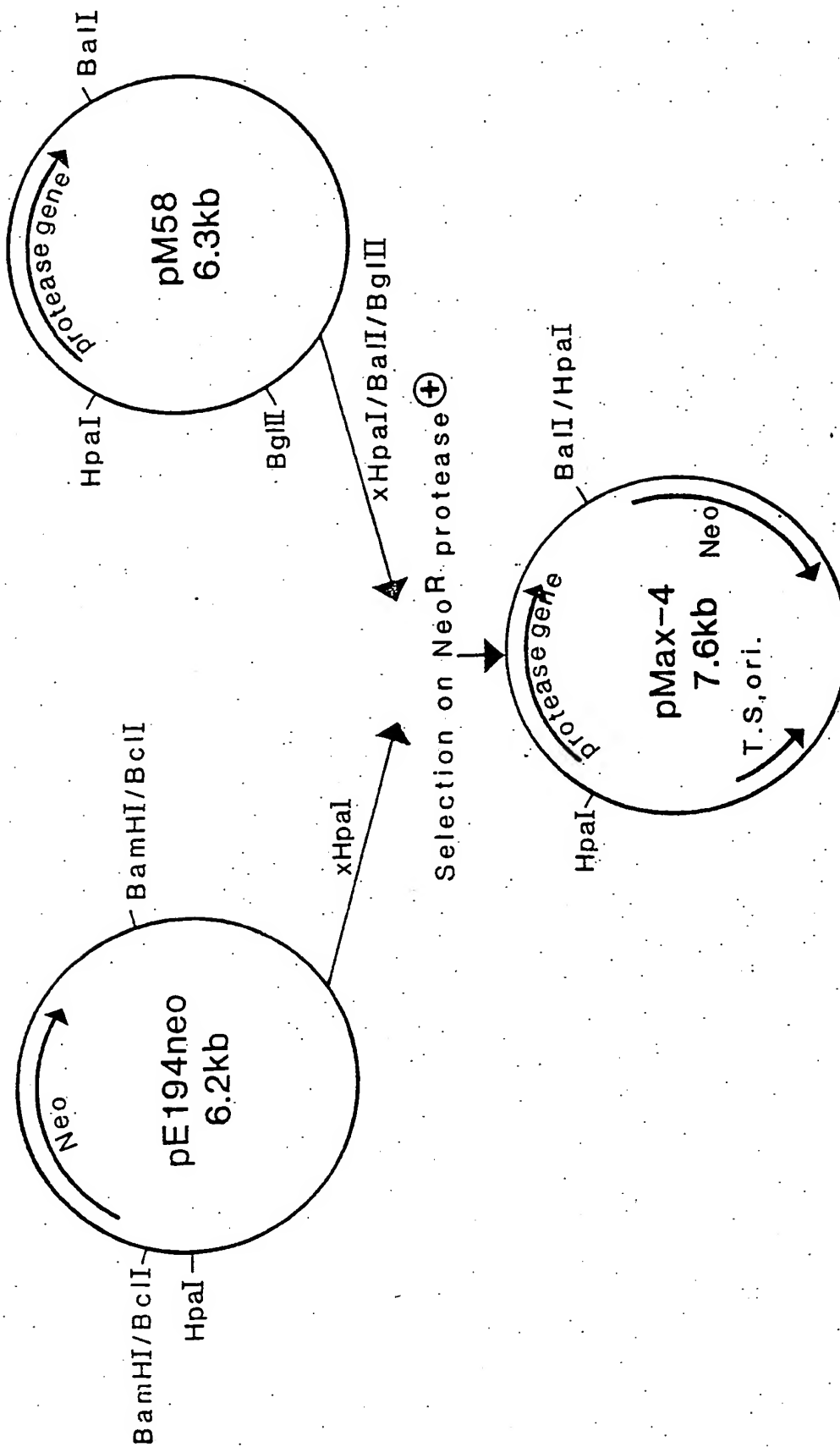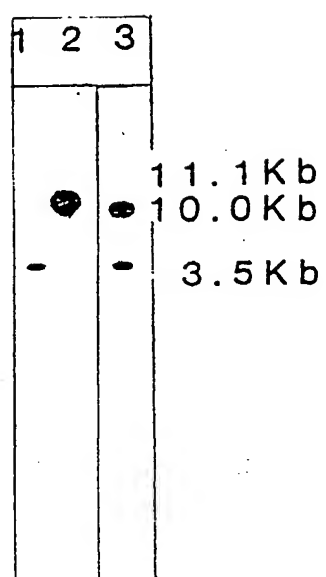terminator

FIGURE 5 (contn'd)

FIGURE 6A

FIGURE 6B

Lane 1: PB92

Lane 2: PBT 109

Lane 3: PBT 108

FIGURE 7A

Protease gene

Neomycin resistance gene

Plasmid sequence

PB92 chromosomal DNA

HindⅢ

HindⅢ

HindⅢ

X

pMAX-4

Homologous recombination

HindⅢ

HindⅢ
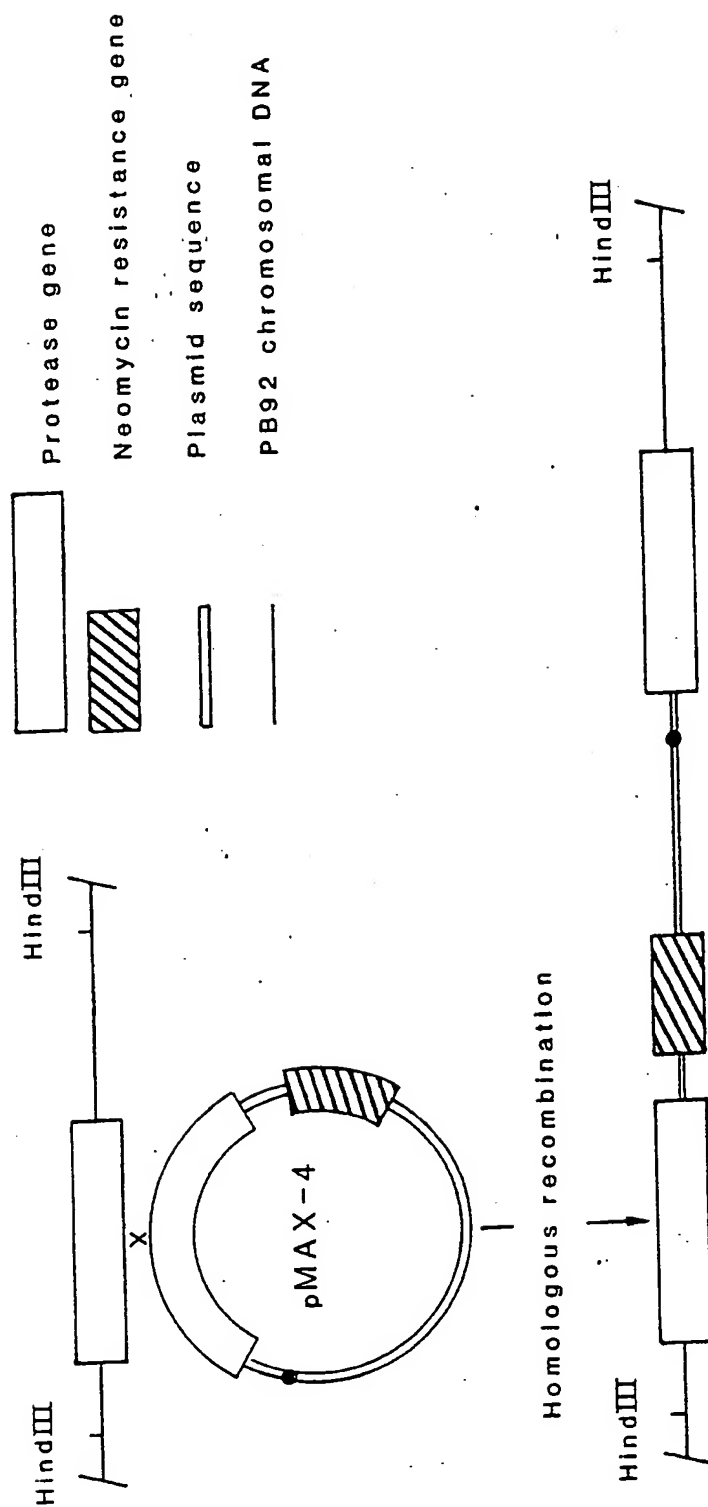
Situation PBT109

FIGURE 7B
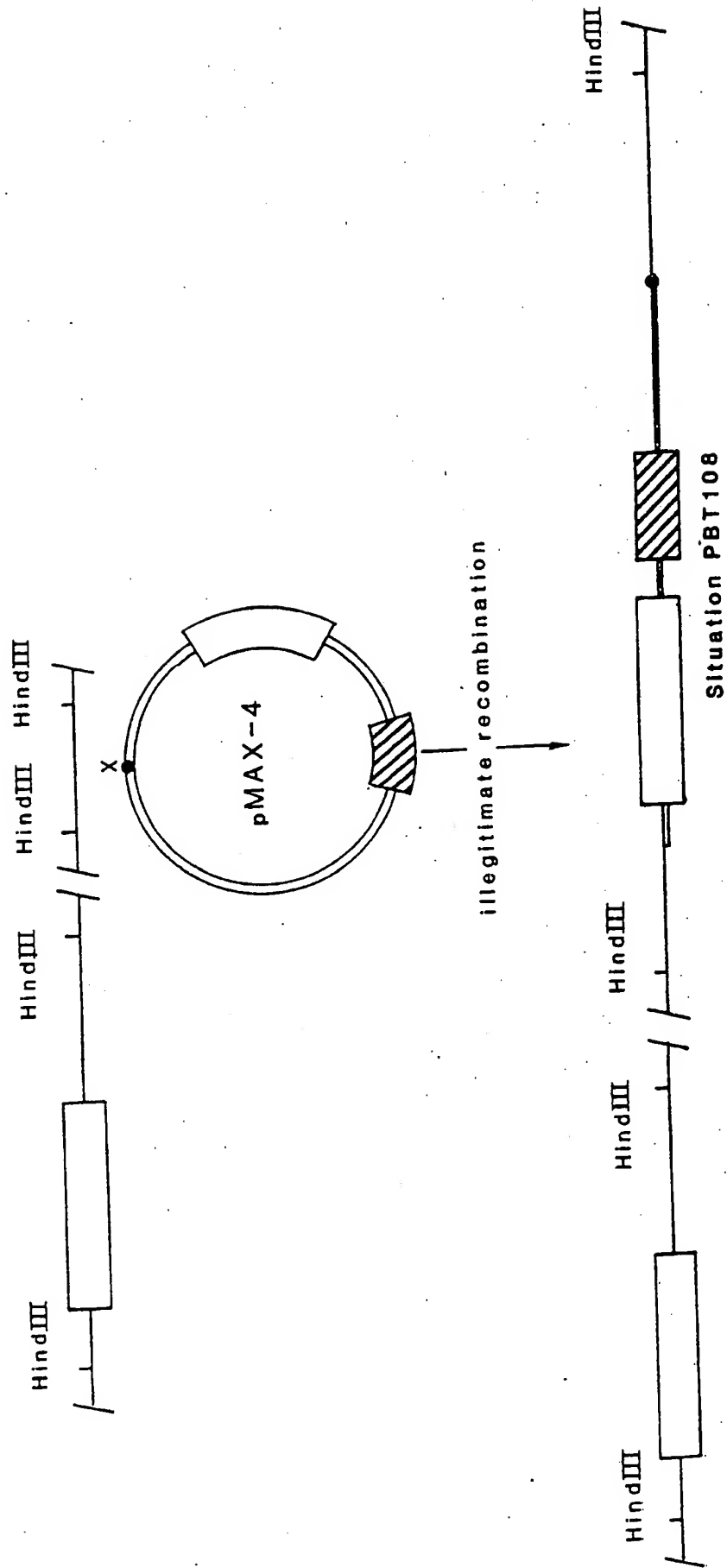
FIGURE 7C

CONSTRUCTION OF INTEGRATION VECTOR pELatB

BglI · Amylase gene · BclI

pGB34
6.6kb

BglII

HpaI · NEO

pE194Neo
6.2kb

T.S.ori.

xBclI, BglI, BglII   xHpaI
made blunt.

Transformation to
B. subtilis 1-A40 selection on NEO[R] AMY[+]
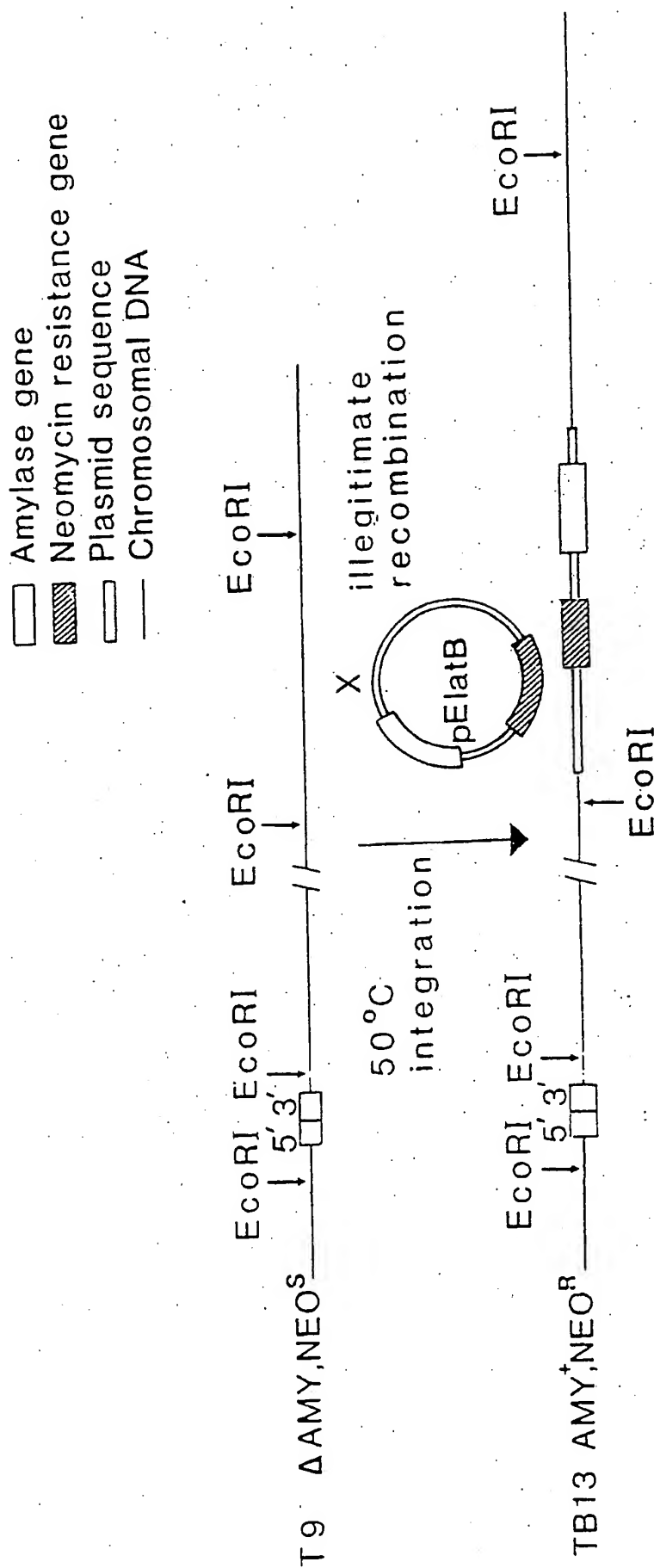
Amylase gene
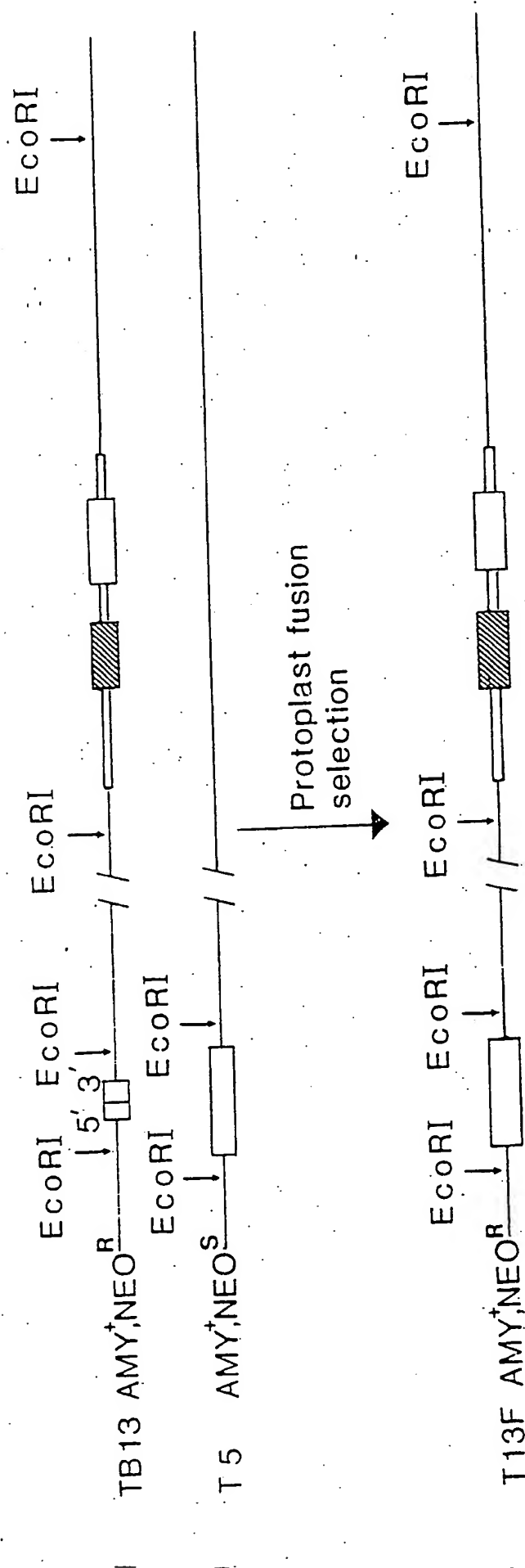
pElatB
8.2kb Neo

T.S.ori.
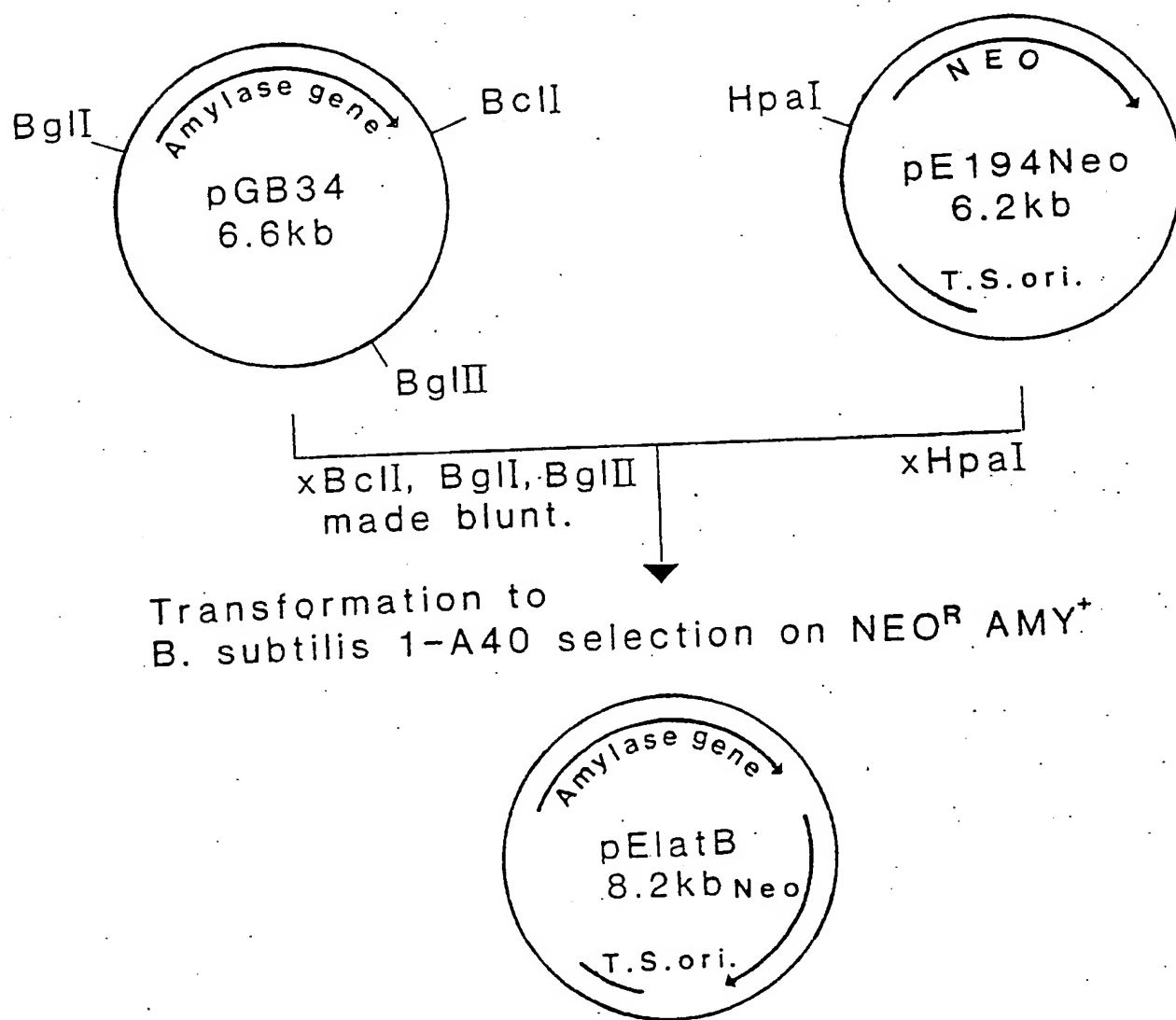
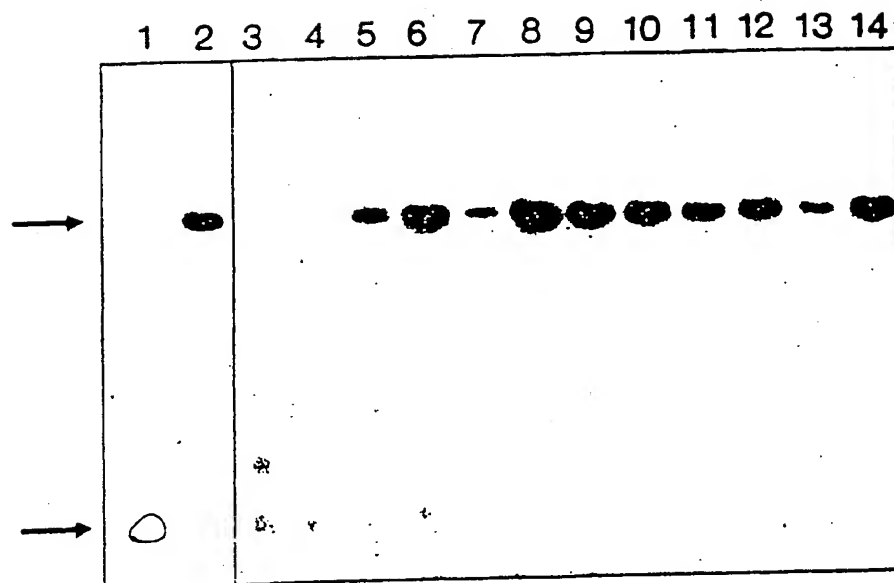FIGURE 8

FIGURE 9A

FIGURE 9B

FIGURE 10

INTERNATIONAL SEARCH REPORT

International Application No PCT/NL 88/00006

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC⁴: C 12 N 15/00; C 12 N 1/20// C 12 N 9/54

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC⁴	C 12 N .

Documentation Searched other than Minimum Documentation
to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category [*]	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	EP, A, 0124374 (GENEX) 7 November 1984, see claims 1-4	1,2,3
Y	see claims 10,11; page 8, lines 10-25	4,6-9,11,13
	--	
X	EP, A, 0205371 (CNRS) 17 December 1986, see column 9, line 8; page 8, table, results QB118::(pCG1)	1-3,7,8,11
	--	
X	Chemical Abstracts, vol. 98, 1983, (Columbus, Ohio, US), J. Hofemeister et al.: "Integration of plasmid pE194 at multiple sites on the Bacillus subtilis chromosome", see page 88, abstract no. 155686a, MGG, Mol. Gen. Genet. 1983, 189(1), 58-68	1
A		14-16
	--	
X	EP, A, 0127328 (GENEX) 5 December 1984, see page 6, line 29 - page 7, line 7; page 7, lines 23-29; page 19, lines 6-11; page 19, line 34 - page 20, line 19 ./.	1-3,7,8,11

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
29th June 1988	0 1 AUG 1988

International Searching Authority	Signature of Authorized Officer
EUROPEAN PATENT OFFICE	M. VAN MOL

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y		14-24,27-30
	--	
X	EP, A, 0074553 (UNIVERSITY OF ROCHESTER) 23 March 1983, see page 16, line 34 - page 18, line 4	1-3,7,8,11
	--	
Y	EP, A, 0134048 (GIST BROCADES) 13 March 1985, see the whole document	4,6-9,11, 13,14-24, 27-30
	--	
X	Chemical Abstracts, vol. 108, 1988, (Columbus, ohio, US), P. Kallio et al.: "Enhancement of alpha-amylase production by integrating and amplifying the alpha-amylase gene of Bacillus amyloliquefaciens in the genome of Bacillus subtilis", see abstract no. 88925n, Appl. Microbiol. Biotechnol. 1987, 27(1), 64-71	1,2,3,7,8, 11
	--	
A	WO, A, 86/01825 (GENEX) 27 March 1986, see page 10, line 15 - page 13, line 27; figure 2; page 16, lines 1-20	14-19,22,23
	--	
A	FR, A, 2292042 (GIST BROCADES) 18 June 1976	10,24,25
	--	
A	EP, A, 0130756 (GENENTECH) 9 January 1985, see page 4, lines 28-35; page 6, lines 21-25; page 10, lines 26-35; page 13, lines 1-8; pages 24-29; figures 1,7	7,8,9,10, 22,23,24, 25
	--	
A	Biological Abstracts, vol. 82, 1986, Biosciences Information Service (Philadelphia, US), J.H. Yu et al.: "Physiological properties and transformation of alkaline-tolerant bacteria", abstract no. 86427, J. Appl. Microbiol. bioeng 14(3): 239-244, 1986	4,20
	--	
A	EP, A, 0032238 (UNIVERSITY OF ROCHESTER) 22 July 1981, see page 8, lines 1-13	
	--	
A	Gene, vol. 24, 28 September 1983, Elsevier Science Publishers (Amsterdam, NL), J.G.K. Williams et al.: "Stable integration of foreign DNA into the chromosome of the cyanobacterium Synechococcus R2", pages 37-51, see abstract; page 49, last paragraph	
	--	
E	WO, A, 87/04461 (AMGEN) 30 July 1987, see page 30, lines 3-6	1-3,7,8,9

ANNEX TO THE INTERNATIONAL SEARCH REPORT
ON INTERNATIONAL PATENT APPLICATION NO.   NL 8800006
SA   21025

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on 25/07/88
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
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